Methods and Composition for Detecting Targets

I. Cross Ref rence to R lated Application

[001] This application claims the benefit under 35 U.S.C. § 119(e) of prior U.S. Provisional Patent Application No. 60/412,189, filed September 19, 2002, which is incorporated herein by reference.

II. Field of the Invention

[002] The invention relates to methods and compositions for the detection of targets in a sample.

III. Background

[003] The detection of the presence or absence of (or quantity of) one or more target sequences in a sample containing one or more target sequences is commonly practiced. For example, the detection of cancer and many infectious diseases, such as AIDS and hepatitis, routinely includes screening biological samples for the presence or absence of diagnostic nucleic acid sequences. Also, detecting the presence or absence of nucleic acid sequences is often used in forensic science, paternity testing, genetic counseling, and organ transplantation.

[004] An organism's genetic makeup is determined by the genes contained within the genome of that organism. Genes are composed of long strands or deoxyribonucleic acid (DNA) polymers that encode the information needed to make proteins. Properties, capabilities, and traits of an organism often are related to the types and amounts of proteins that are, or are not, being produced by that organism.

[005] A protein can be produced from a gene as follows. First, the DNA of the gene that encodes a protein, for example, protein "X", is converted into ribonucleic acid (RNA) by a process known as "transcription." During transcription, a single-stranded complementary RNA copy of the gene is made. Next, this RNA copy, referred to as protein X messenger RNA (mRNA), is used by the cell's biochemical machinery to make protein X, a process referred to as "translation." Basically, the cell's protein manufacturing machinery binds to the mRNA, "reads" the RNA code, and "translates" it into the amino acid sequence of protein X. In summary, DNA is transcribed to make mRNA, which is translated to make proteins.

[006] The amount of protein X that is produced by a cell often is largely dependent on the amount of protein X mRNA that is present within the cell. The amount of protein X mRNA within a cell is due, at least in part, to the degree to which gene X is expressed. Whether a particular gene is expressed, and if so, to what level, may have a significant impact on the organism.

IV. Summary of the Invention

[007] In certain embodiments, methods are provided for detecting at least one target nucleic acid sequence in a sample. In certain embodiments, the methods comprise forming a ligation reaction composition comprising the sample and a ligation probe set for each target nucleic acid sequence. In certain embodiments, the probe set comprises (a) at least one first probe, comprising a target-specific portion and a 5' primer-specific portion, wherein the 5' primer-specific portion comprises a sequence, and (b) at least one second probe, comprising a target-specific portion and a 3' primer-specific portion, wherein the 3' primer-specific portion comprises a sequence. In certain embodiments, the probes in each set are suitable for ligation together when hybridized adjacent to one

another on a complementary target nucleic acid sequence, and one probe in each probe set further comprises an addressable portion located between the primer-specific portion and the target-specific portion, wherein the addressable portion comprises a sequence.

[008] In certain embodiments, the methods further comprise forming a test composition by subjecting the ligation reaction composition to at least one cycle of ligation, wherein adjacently hybridizing complementary probes are ligated to one another to form a ligation product comprising the 5' primer-specific portion, the target-specific portions, the addressable portion, and the 3' primer-specific portion.

[009] In certain embodiments, the methods further comprise forming an amplification reaction composition comprising:

the test composition;

a polymerase;

a labeled probe, wherein the labeled probe has a first detectable signal value when it is not hybridized to a complementary sequence, and wherein the labeled probe comprises the sequence of the addressable portion or comprises a sequence complementary to the sequence of the addressable portion; and

at least one primer set, the primer set comprising (i) at least one first primer comprising the sequence of the 5' primer-specific portion of the ligation product, and (ii) at least one second primer comprising a sequence complementary to the sequence of the 3' primer-specific portion of the ligation product.

[010] In certain embodiments, the methods further comprise subjecting the amplification reaction composition to at least one amplification reaction. In certain embodiments, the methods further comprise detecting a second detectable signal value at least one of during and after the amplification reaction, wherein a threshold

difference between the first detectable signal value and the second detectable signal value indicates the presence of the target nucleic acid sequence, and wherein no threshold difference between the first detectable signal value and the second detectable signal value indicates the absence of the target nucleic acid sequence.

[011] In certain embodiments, methods are provided for detecting at least one target nucleic acid sequence in a sample. In certain embodiments, the methods comprise forming a ligation reaction composition comprising the sample and a ligation probe set for each target nucleic acid sequence. In certain embodiments, the probe set comprises (a) at least one first probe, comprising a target-specific portion and a 5' primer-specific portion, wherein the 5' primer-specific portion comprises a sequence, and (b) at least one second probe, comprising a target-specific portion and a 3' primer-specific portion, wherein the 3' primer-specific portion comprises a sequence. In certain embodiments, the probes in each set are suitable for ligation together when hybridized adjacent to one another on a complementary target nucleic acid sequence, and one probe in each probe set further comprises an addressable portion located between the primer-specific portion and the target-specific portion, wherein the addressable portion comprises a sequence.

[012] In certain embodiments, the methods further comprise forming a test composition by subjecting the ligation reaction composition to at least one cycle of ligation, wherein adjacently hybridizing complementary probes are ligated to one another to form a ligation product comprising the 5' primer-specific portion, the target-specific portions, the addressable portion, and the 3' primer-specific portion.

[013] In certain embodiments, the methods further comprise forming an amplification reaction composition comprising:

the test composition;

a polymerase;

a labeled probe, wherein the labeled probe has a first detectable signal value when it is not hybridized to a complementary sequence, and wherein the labeled probe comprises the sequence of the addressable portion or comprises a sequence complementary to the sequence of the addressable portion; and

at least one primer set, the primer set comprising (i) at least one first primer comprising the sequence of the 5' primer-specific portion of the ligation product, and (ii) at least one second primer comprising a sequence complementary to the sequence of the 3' primer-specific portion of the ligation product.

[014] In certain embodiments, the methods further comprise subjecting the amplification reaction composition to at least one amplification reaction. In certain embodiments, the methods further comprise detecting the presence or absence of the target nucleic acid sequence by monitoring a signal at least one of during and after the at least one amplification reaction.

[015] In certain embodiments, kits are provided for detecting at least one target nucleic acid sequence. In certain embodiments, the kits comprise a ligation probe set for each target nucleic acid sequence. In certain embodiments, the probe set comprises (a) at least one first probe, comprising a target-specific portion and a 5' primer-specific portion, wherein the 5' primer-specific portion comprises a sequence, and (b) at least one second probe, comprising a target-specific portion and a 3' primer-specific portion, wherein the 3' primer-specific portion comprises a sequence. In certain embodiments, the probes in each set are suitable for ligation

together when hybridized adjacent to one another on a complementary target nucleic acid sequence, and one probe in each probe set further comprises an addressable portion located between the primer-specific portion and the target-specific portion, wherein the addressable portion comprises a sequence.

[016] In certain embodiments, the kits further comprise a labeled probe comprising the sequence of the addressable portion or comprising a sequence complementary to the sequence of the addressable portion.

V. Brief Description of the Drawings

[017] The skilled artisan will understand that the drawings, described below, are for illustration purposes only. The figures are not intended to limit the scope of the invention in any way.

[018] Figure 1. Schematic showing of labeled probes according to certain exemplary embodiments.

[019] Figure 2 (2A-2E). Schematic showing an exemplary embodiment of certain embodiments comprising ligation and primer extension amplification.

[020] Figure 3 (3A-3F) depicts exemplary embodiments of the invention comprising ligation and PCR-based amplification, wherein the exemplary target nucleic acid sequence is an mRNA in the sample.

[021] Figure 4 is a schematic showing a ligation probe set according to certain embodiments of the invention.

[022] Each probe includes a portion that is complementary to the target (the "target-specific portion," T-SP) and a portion that is complementary to or has the same sequence as a primer (the "primer-specific portion," P-SP). At least one probe in each probe set further comprises an addressable portion (ASP) that is

located between the target-specific portion and the primer-specific portion (here, the second probe).

[023] Each probe set comprises at least one first probe and at least one second probe that are designed to hybridize with the target with the 3' end of the first probe (here, probe A) immediately adjacent to and opposing the 5' end of the second probe (here, probe Z).

[024] Figure 5 depicts a method for differentiating between two potential alleles in a target locus using certain embodiments of the invention.

[025] Fig. 5 at (1) shows: (i) a target-specific probe set comprising: two first probes (A and B) that have the same primer-specific portions (P-SP1), the same target-specific portions except for different pivotal complements (here, T at the 3' end probe A and C at the 3' end probe B) and different addressable portions ((ASP-A) and (ASP-B)); and one second probe (Z) comprising a target-specific portion and a primer-specific portion (P-SP2).

[026] Fig. 5 at (2) shows the three probes annealed to the target. The target-specific portion of probe A is fully complementary with the 3' target region including the pivotal nucleotide. The pivotal complement of probe B is not complementary with the 3' target region. The target-specific portion of probe B, therefore, contains a base-pair mismatch at the 3' end. The target-specific portion of probe Z is fully complementary to the 5' target region.

[027] Fig. 5 at (3) shows ligation of probes A and Z to form ligation product A-Z. Probes B and Z are not ligated together to form a ligation product due to the mismatched pivotal complement on probe B.

[028] Fig. 5 at (4) shows denaturing the double-stranded molecules to release the A-Z ligation product and unligated probes B and Z.

[029] Figure 6 is a schematic depicting certain embodiments of the invention.

[030] Fig. 6(A) at (1) depicts a target sequence and a ligation probe set comprising: two first probes (A and B) that have the same primer-specific portions (P-SP1), the same target-specific portions except for different pivotal complements (here, T at the 3' end probe A and G at the 3'end probe B) and different addressable portions ((ASP-A) and (ASP-B)); and one second probe (Z) comprising a target-specific portion and a primer-specific portion (P-SP2).

[031] Fig. 6(A) at (2) depicts the A and Z probes hybridized to the target sequence under annealing conditions.

[032] Fig. 6(A) at (3) depicts the ligation of the first and second probes in the presence of a ligation agent to form ligation product.

[033] Fig. 6(A) at (4) depicts denaturing the ligation product:target complex to release a single-stranded ligation product; adding a primer set (P1 and P2) and two labeled probes (LBP-A and LBP-B); and annealing primer P2 to the ligation product .

[034] Fig. 6(A) at (5) depicts the formation of a double-stranded nucleic acid product by extending the P2 primer in a template-dependent manner with a polymerase.

[035] Figs. 6B and 6C at (6) through 11 depict additional cycles of amplification.

[036] Figure 7 (7A-7C) depicts certain embodiments involving three biallelic loci.

[037] Figure 8 depicts certain embodiments employing flap endonuclease.

VI. Detailed Description of Certain Exemplary Embodiments

[038] It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. In this application, the use of the singular includes the plural unless specifically stated otherwise. In this application, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including", as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one subunit unless specifically stated otherwise.

[039] The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including but not limited to patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference in their entirety for any purpose. U.S. Patent Application Serial Nos. 09/584,905, filed May 30, 2000, 09/724,755, filed November 28, 2000, 10/011,993, filed December 5, 2001, and Patent Cooperation Treaty Application No. PCT/US01/17329, filed May 30, 2001, are hereby expressly incorporated by reference in their entirety for any purpose.

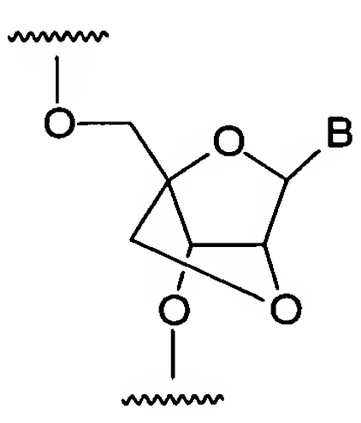
A. Certain Definitions

[040] The term "nucleotide base", as used herein, refers to a substituted or unsubstituted aromatic ring or rings. In certain embodiments, the aromatic ring or rings contain at least one nitrogen atom. In certain embodiments, the nucleotide base is capable of forming Watson-Crick and/or Hoogsteen hydrogen bonds with

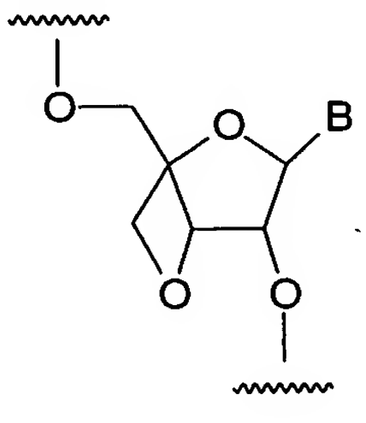
an appropriately complementary nucleotide base. Exemplary nucleotide bases and analogs thereof include, but are not limited to, naturally occurring nucleotide bases adenine, guanine, cytosine, 6 methyl-cytosine, uracil, thymine, and analogs of the naturally occurring nucleotide bases, e.g., 7-deazaadenine, 7-deazaguanine, 7-deaza-8-azaguanine, 7-deaza-8-azaadenine, N6 -Δ2 -isopentenyladenine (6iA), N6 -Δ2 -isopentenyl-2-methylthioadenine (2ms6iA), N2 -dimethylguanine (dmG), 7-methylguanine (7mG), inosine, nebularine, 2-aminopurine, 2-amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, pseudouridine, pseudocytosine, pseudoisocytosine, 5-propynylcytosine, isocytosine, isoguanine, 7-deazaguanine, 2-thiopyrimidine, 6-thioguanine, 4-thiothymine, 4-thiouracil, O^6-methylguanine, N^6-methyladenine, O^4-methylthymine, 5,6-dihydrothymine, 5,6-dihydrouracil, pyrazolo[3,4-D]pyrimidines (see, e.g., U.S. Patent Nos. 6,143,877 and 6,127,121 and PCT published application WO 01/38584), ethenoadenine, indoles such as nitroindole and 4-methylindole, and pyrroles such as nitropyrrole. Certain exemplary nucleotide bases can be found, e.g., in Fasman, 1989, Practical Handbook of Biochemistry and Molecular Biology, pp. 385-394, CRC Press, Boca Raton, Fla., and the references cited therein.

[041] The term “nucleotide”, as used herein, refers to a compound comprising a nucleotide base linked to the C-1' carbon of a sugar, such as ribose, arabinose, xylose, and pyranose, and sugar analogs thereof. The term nucleotide also encompasses nucleotide analogs. The sugar may be substituted or unsubstituted. Substituted ribose sugars include, but are not limited to, those riboses in which one or more of the carbon atoms, for example the 2'-carbon atom, is substituted with one or more of the same or different Cl, F, –R, –OR, $–NR_2$ or halogen groups, where each R is independently H, C_1-C_6 alkyl or C_5-C_{14} aryl.

Exemplary riboses include, but are not limited to, 2'-(C1 -C6)alkoxyribose, 2'-(C5 -C14)aryloxyribose, 2',3'-didehydroribose, 2'-deoxy-3'-haloribose, 2'-deoxy-3'-fluororibose, 2'-deoxy-3'-chlororibose, 2'-deoxy-3'-aminoribose, 2'-deoxy-3'-(C1 -C6)alkylribose, 2'-deoxy-3'-(C1 -C6)alkoxyribose and 2'-deoxy-3'-(C5 -C14)aryloxyribose, ribose, 2'-deoxyribose, 2',3'-dideoxyribose, 2'-haloribose, 2'-fluororibose, 2'-chlororibose, and 2'-alkylribose, e.g., 2'-O-methyl, 4'-α-anomeric nucleotides, 1'-α-anomeric nucleotides, 2'-4'- and 3'-4'-linked and other "locked" or "LNA", bicyclic sugar modifications (see, e.g., PCT published application nos. WO 98/22489, WO 98/39352;, and WO 99/14226). Exemplary LNA sugar analogs within a polynucleotide include, but are not limited to, the structures:



2'-4' LNA



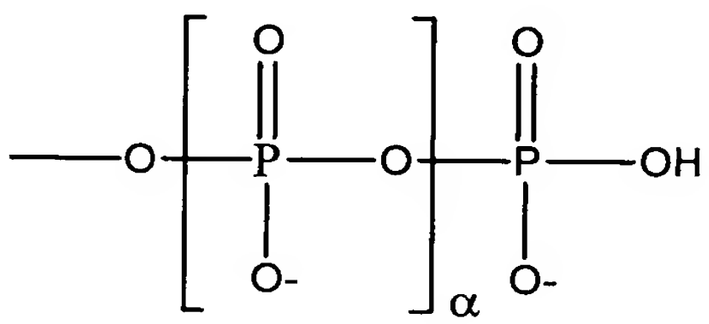
3'-4' LNA

where B is any nucleotide base.

[042] Modifications at the 2'- or 3'-position of ribose include, but are not limited to, hydrogen, hydroxy, methoxy, ethoxy, allyloxy, isopropoxy, butoxy, isobutoxy, methoxyethyl, alkoxy, phenoxy, azido, amino, alkylamino, fluoro, chloro and bromo. Nucleotides include, but are not limited to, the natural D optical isomer, as well as the L optical isomer forms (see, e.g., Garbesi (1993) Nucl. Acids Res. 21:4159-65; Fujimori (1990) J. Amer. Chem. Soc. 112:7435; Urata, (1993) Nucleic Acids Symposium Ser. No. 29:69-70). When the nucleotide base is purine, e.g. A or G, the ribose sugar is attached to the N^9-position of the nucleotide base. When the nucleotide base is pyrimidine, e.g. C, T or U, the pentose sugar is attached to

the N^1-position of the nucleotide base, except for pseudouridines, in which the pentose sugar is attached to the C5 position of the uracil nucleotide base (see, e.g., Kornberg and Baker, (1992) *DNA Replication*, 2nd Ed., Freeman, San Francisco, CA).

[043] One or more of the pentose carbons of a nucleotide may be substituted with a phosphate ester having the formula:



where α is an integer from 0 to 4. In certain embodiments, α is 2 and the phosphate ester is attached to the 3'- or 5'-carbon of the pentose. In certain embodiments, the nucleotides are those in which the nucleotide base is a purine, a 7-deazapurine, a pyrimidine, or an analog thereof. "Nucleotide 5'-triphosphate" refers to a nucleotide with a triphosphate ester group at the 5' position, and are sometimes denoted as "NTP", or "dNTP" and "ddNTP" to particularly point out the structural features of the ribose sugar. The triphosphate ester group may include sulfur substitutions for the various oxygens, e.g. α-thio-nucleotide 5'-triphosphates. For a review of nucleotide chemistry, see: Shabarova, Z. and Bogdanov, A. *Advanced Organic Chemistry of Nucleic Acids*, VCH, New York, 1994.

[044] The term "nucleotide analog", as used herein, refers to embodiments in which the pentose sugar and/or the nucleotide base and/or one or more of the phosphate esters of a nucleotide may be replaced with its respective analog. In certain embodiments, exemplary pentose sugar analogs are those described above. In certain embodiments, the nucleotide analogs have a nucleotide base

analog as described above. In certain embodiments, exemplary phosphate ester analogs include, but are not limited to, alkylphosphonates, methylphosphonates, phosphoramidates, phosphotriesters, phosphorothioates, phosphorodithioates, phosphoroselenoates, phosphorodiselenoates, phosphoroanilothioates, phosphoroanilidates, phosphoroamidates, boronophosphates, etc., and may include associated counterions.

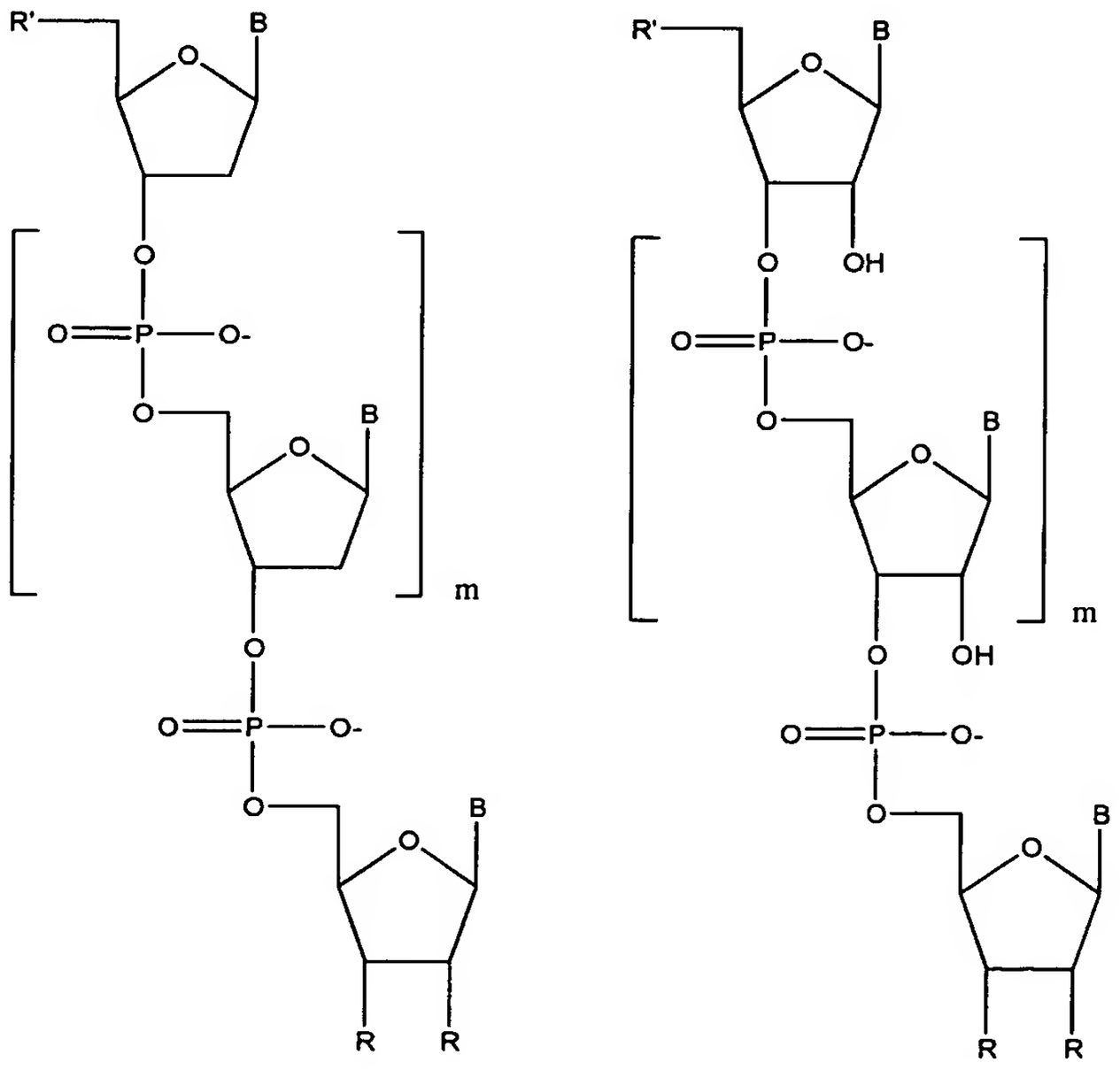
[045] Also included within the definition of "nucleotide analog" are nucleotide analog monomers which can be polymerized into polynucleotide analogs in which the DNA/RNA phosphate ester and/or sugar phosphate ester backbone is replaced with a different type of internucleotide linkage. Exemplary polynucleotide analogs include, but are not limited to, peptide nucleic acids, in which the sugar phosphate backbone of the polynucleotide is replaced by a peptide backbone.

[046] As used herein, the terms "polynucleotide", "oligonucleotide", and "nucleic acid" are used interchangeably and mean single-stranded and double-stranded polymers of nucleotide monomers, including 2'-deoxyribonucleotides (DNA) and ribonucleotides (RNA) linked by internucleotide phosphodiester bond linkages, or internucleotide analogs, and associated counter ions, e.g., H^+, NH_4^+, trialkylammonium, Mg^{2+}, Na^+ and the like. A nucleic acid may be composed entirely of deoxyribonucleotides, entirely of ribonucleotides, or chimeric mixtures thereof. The nucleotide monomer units may comprise any of the nucleotides described herein, including, but not limited to, naturally occuring nucleotides and nucleotide analogs. nucleic acids typically range in size from a few monomeric units, e.g. 5-40 when they are sometimes referred to in the art as oligonucleotides, to several thousands of monomeric nucleotide units. Unless denoted otherwise, whenever a nucleic acid sequence is represented, it will be understood that the nucleotides are in

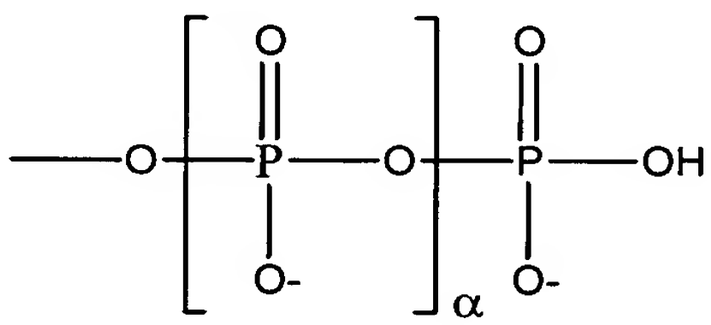
5' to 3' order from left to right and that "A" denotes deoxyadenosine or an analog thereof, "C" denotes deoxycytidine or an analog thereof, "G" denotes deoxyguanosine or an analog thereof, and "T" denotes thymidine or an analog thereof, unless otherwise noted.

[047] Nucleic acids include, but are not limited to, genomic DNA, cDNA, hnRNA, mRNA, rRNA, tRNA, fragmented nucleic acid, nucleic acid obtained from subcellular organelles such as mitochondria or chloroplasts, and nucleic acid obtained from microorganisms or DNA or RNA viruses that may be present on or in a biological sample.

[048] Nucleic acids may be composed of a single type of sugar moiety, e.g., as in the case of RNA and DNA, or mixtures of different sugar moieties, e.g., as in the case of RNA/DNA chimeras. In certain embodiments, nucleic acids are ribopolynucleotides and 2'-deoxyribopolynucleotides according to the structural formulae below:



[049] wherein each B is independently the base moiety of a nucleotide, e.g., a purine, a 7-deazapurine, a pyrimidine, or an analog nucleotide; each m defines the length of the respective nucleic acid and can range from zero to thousands, tens of thousands, or even more; each R is independently selected from the group comprising hydrogen, halogen, --R", --OR", and --NR"R", where each R" is independently (C1 -C6) alkyl or (C5 -C14) aryl, or two adjacent Rs are taken together to form a bond such that the ribose sugar is 2',3'-didehydroribose; and each R' is independently hydroxyl or



where α is zero, one or two.

[050] In certain embodiments of the ribopolynucleotides and 2'-deoxyribopolynucleotides illustrated above, the nucleotide bases B are covalently attached to the C1' carbon of the sugar moiety as previously described.

[051] The terms “nucleic acid”, “polynucleotide”, and “oligonucleotide” may also include nucleic acid analogs, polynucleotide analogs, and oligonucleotide analogs. The terms “nucleic acid analog”, "polynucleotide analog" and “oligonucleotide analog” are used interchangeably and, as used herein, refer to a nucleic acid that contains at least one nucleotide analog and/or at least one phosphate ester analog and/or at least one pentose sugar analog. Also included within the definition of nucleic acid analogs are nucleic acids in which the phosphate

ester and/or sugar phosphate ester linkages are replaced with other types of linkages, such as N-(2-aminoethyl)-glycine amides and other amides (see, e.g., Nielsen et al., 1991, *Science* **254**: 1497-1500; WO 92/20702; U.S. Pat. No. 5,719,262; U.S. Pat. No. 5,698,685;); morpholinos (see, e.g., U.S. Pat. No. 5,698,685; U.S. Pat. No. 5,378,841; U.S. Pat. No. 5,185,144); carbamates (see, e.g., Stirchak & Summerton, 1987, *J. Org. Chem.* **52**: 4202); methylene(methylimino) (see, e.g., Vasseur et al., 1992, *J. Am. Chem. Soc.* **114**: 4006); 3'-thioformacetals (see, e.g., Jones et al., 1993, *J. Org. Chem.* **58**: 2983); sulfamates (see, e.g., U.S. Pat. No. 5,470,967); 2-aminoethylglycine, commonly referred to as PNA (see, e.g., Buchardt, WO 92/20702; Nielsen (1991) Science 254:1497-1500); and others (see, e.g., U.S. Pat. No. 5,817,781; Frier & Altman, 1997, *Nucl. Acids Res.* **25**:4429 and the references cited therein). Phosphate ester analogs include, but are not limited to, (i) C_1–C_4 alkylphosphonate, e.g. methylphosphonate; (ii) phosphoramidate; (iii) C_1–C_6 alkyl-phosphotriester; (iv) phosphorothioate; and (v) phosphorodithioate.

[052] The terms "annealing" and "hybridization" are used interchangeably and mean the base-pairing interaction of one nucleic acid with another nucleic acid that results in formation of a duplex, triplex, or other higher-ordered structure. In certain embodiments, the primary interaction is base specific, e.g., A/T and G/C, by Watson/Crick and Hoogsteen-type hydrogen bonding. In certain embodiments, base-stacking and hydrophobic interactions may also contribute to duplex stability.

[053] An “enzymatically active mutant or variant thereof,” when used in reference to an enzyme such as a polymerase or a ligase, means a protein with appropriate enzymatic activity. Thus, for example, but without limitation, an enzymatically active mutant or variant of a DNA polymerase is a protein that is able to

catalyze the stepwise addition of appropriate deoxynucleoside triphosphates into a nascent DNA strand in a template-dependent manner. An enzymatically active mutant or variant differs from the "generally-accepted" or consensus sequence for that enzyme by at least one amino acid, including, but not limited to, substitutions of one or more amino acids, addition of one or more amino acids, deletion of one or more amino acids, and alterations to the amino acids themselves. With the change, however, at least some catalytic activity is retained. In certain embodiments, the changes involve conservative amino acid substitutions. Conservative amino acid substitution may involve replacing one amino acid with another that has, e.g., similar hydorphobicity, hydrophilicity, charge, or aromaticity. In certain embodiments, conservative amino acid substitutions may be made on the basis of similar hydropathic indices. A hydropathic index takes into account the hydrophobicity and charge characteristics of an amino acid, and in certain embodiments, may be used as a guide for selecting conservative amino acid substitutions. The hydropathic index is discussed, e.g., in Kyte *et al.*, *J. Mol. Biol.*, 157:105-131 (1982). It is understood in the art that conservative amino acid substitutions may be made on the basis of any of the aforementioned characteristics.

[054] Alterations to the amino acids may include, but are not limited to, glycosylation, methylation, phosphorylation, biotinylation, and any covalent and noncovalent additions to a protein that do not result in a change in amino acid sequence. "Amino acid" as used herein refers to any amino acid, natural or nonnatural, that may be incorporated, either enzymatically or synthetically, into a polypeptide or protein.

[055] Fragments, for example, but without limitation, proteolytic cleavage products, are also encompassed by this term, provided that at least some enzyme catalytic activity is retained.

[056] The skilled artisan will readily be able to measure catalytic activity using an appropriate well-known assay. Thus, an appropriate assay for polymerase catalytic activity might include, for example, measuring the ability of a variant to incorporate, under appropriate conditions, rNTPs or dNTPs into a nascent polynucleotide strand in a template-dependent manner. Likewise, an appropriate assay for ligase catalytic activity might include, for example, the ability to ligate adjacently hybridized oligonucleotides comprising appropriate reactive groups. Protocols for such assays may be found, among other places, in Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press (1989) (hereinafter "Sambrook et al."), Sambrook and Russell, Molecular Cloning, Third Edition, Cold Spring Harbor Press (2000) (hereinafter "Sambrook and Russell"), Ausbel et al., Current Protocols in Molecular Biology (1993) including supplements through April 2001, John Wiley & Sons (hereinafter "Ausbel et al.").

[057] A "target" or "target nucleic acid sequence" according to the present invention comprises a specific nucleic acid sequence that can be distinguished by a probe. Targets may include both naturally occurring and synthetic molecules.

[058] "Probes", according to the present invention, comprise oligonucleotides that comprise a specific portion that is designed to hybridize in a sequence-specific manner with a complementary region on a specific nucleic acid sequence, e.g., a target nucleic acid sequence. In certain embodiments, the specific portion of the probe may be specific for a particular sequence, or alternatively, may be degenerate, e.g., specific for a set of sequences.

[059] A "ligation probe set" according to the present invention is a group of two or more probes designed to detect at least one target. As a non-limiting example, a ligation probe set may comprise two nucleic acid probes designed to hybridize to a target such that, when the two probes are hybridized to the target adjacent to one another, they are suitable for ligation together.

[060] When used in the context of the present invention, "suitable for ligation" refers to at least one first target-specific probe and at least one second target-specific probe, each comprising an appropriately reactive group. Exemplary reactive groups include, but are not limited to, a free hydroxyl group on the 3' end of the first probe and a free phosphate group on the 5' end of the second probe. Exemplary pairs of reactive groups include, but are not limited to: phosphorothioate and tosylate or iodide; esters and hydrazide; $RC(O)S^-$, haloalkyl, or RCH_2S and α-haloacyl; thiophosphoryl and bromoacetoamido groups. Exemplary reactive groups include, but are not limited to, S-pivaloyloxymethyl-4-thiothymidine. Additionally, in certain embodiments, first and second target-specific probes are hybridized to the target sequence such that the 3' end of the first target-specific probe and the 5' end of the second target-specific probe are immediately adjacent to allow ligation.

[061] The term "signal moiety" as used herein refers to any tag, label, or identifiable moiety.

[062] "Detectably different signal" means that detectable signals from different signal moieties are distinguishable from one another by at least one detection method.

[063] The term "detectable signal value" refers to a value of the signal that is detected from a label. In certain embodiments, the detectable signal value is the amount or intensity of signal that is detected from a label. Thus, if there is no

detectable signal value from a label, its detectable signal value is zero (0). In certain embodiments, the detectable signal value is a characteristic of the signal other than the amount or intensity of the signal, such as the spectra, wavelength, color, or lifetime of the signal.

[064] "Detectably different signal value" means that one or more detectable signal values are distinguishable from one another by at least one detection method.

[065] The term "labeled probe" refers to a probe that provides a detectably different signal value depending upon whether a given nucleic acid sequence is present or absent. In certain embodiments, a labeled probe provides a detectably different signal value when the intact labeled probe is hybridized to a given nucleic acid sequence than when the intact labeled probe is not hybridized to a given nucleic acid sequence. Thus, if a given nucleic acid sequence is present, the labeled probe provides a detectably different signal value than when the given nucleic acid sequence is absent. In certain embodiments, a labeled probe provides a detectably different signal value when the probe is intact than when the probe is not intact. In certain such embodiments, a labeled probe remains intact unless a given nucleic acid sequence is present. In certain such embodiments, if a given nucleic acid sequence is present, the labeled probe is cleaved, which results in a detectably different signal value than when the probe is intact.

[066] In certain embodiments, the labeled probe is an "interaction probe." The term "interaction probe" refers to a probe that comprises at least two moieties that can interact with one another to provide a detectably different signal value depending upon whether a given nucleic acid sequence is present or absent. The signal value that is detected from the interaction probe is different depending on

whether the two moieties are sufficiently close to one another or are spaced apart from one another. During the methods described herein, the proximity of the two moieties to one another is different depending upon whether the given nucleic acid is present or absent.

[067] In certain embodiments, the two moieties of the interaction probe are moved further apart if the given nucleic acid sequence is present. In certain embodiments, the interaction probe comprises two moieties that are linked together by a link element, and the two moieties become unlinked during the method if the given nucleic acid sequence is present. The signal value that is detected from the interaction probe that includes the two moieties linked together is different from the signal value that is detected from the interaction probe when the two moieties are not linked.

[068] The term "threshold difference between signal values" refers to a set difference between a first detectable signal value and a second detectable signal value that results when the target nucleic acid sequence that is being sought is present in a sample, but that does not result when the target nucleic acid sequence is absent. The first detectable signal value of a labeled probe is the detectable signal value from the probe when it is not exposed to a given nucleic acid sequence. The second detectable signal value is detected during and/or after an amplification reaction using a composition that comprises the labeled probe.

[069] The term "quantitating," when used in reference to an amplification product, refers to determining the quantity or amount of a particular sequence that is representative of a target nucleic acid sequence in the sample. For example, but without limitation, one may measure the intensity of the signal from a labeled probe. The intensity or quantity of the signal is typically related to the amount of

amplification product. The amount of amplification product generated correlates with the amount of target nucleic acid sequence present prior to ligation and amplification, and thus, in certain embodiments, may indicate the level of expression for a particular gene.

[070] The term "amplification product" as used herein refers to the product of an amplification reaction including, but not limited to, primer extension, the polymerase chain reaction, RNA transcription, and the like. Thus, exemplary amplification products may comprise at least one of primer extension products, PCR amplicons, RNA transcription products, and the like.

[071] "Primers" according to the present invention refer to oligonucleotides that are designed to hybridize with the primer-specific portion of probes, ligation products, or amplification products in a sequence-specific manner, and serve as primers for amplification reactions.

[072] A "universal primer" is capable of hybridizing to the primer-specific portion of more than one species of probe, ligation product, or amplification product, as appropriate. A "universal primer set" comprises a first primer and a second primer that hybridize with a plurality of species of probes, ligation products, or amplification products, as appropriate.

[073] A "ligation agent" according to the present invention may comprise any number of enzymatic or chemical (i.e., non-enzymatic) agents that can effect ligation of nucleic acids to one another.

[074] In this application, a statement that one sequence is the same as or is complementary to another sequence encompasses situations where both of the sequences are completely the same or complementary to one another, and situations where only a portion of one of the sequences is the same as, or is

complementary to, a portion or the entire other sequence. Here, the term "sequence" encompasses, but is not limited to, nucleic acid sequences, polynucleotides, oligonucleotides, probes, primers, primer-specific portions, target-specific portions, addressable portions, and oligonucleotide link elements.

[075] In this application, a statement that one sequence is complementary to another sequence encompasses situations in which the two sequences have mismatches. Here, the term "sequence" encompasses, but is not limited to, nucleic acid sequences, polynucleotides, oligonucleotides, probes, primers, primer-specific portions, target-specific portions, addressable portions, and oligonucleotide link elements. Despite the mismatches, the two sequences should selectively hybridize to one another under appropriate conditions.

[076] The term "selectively hybridize" means that, for particular identical sequences, a substantial portion of the particular identical sequences hybridize to a given desired sequence or sequences, and a substantial portion of the particular identical sequences do not hybridize to other undesired sequences. A "substantial portion of the particular identical sequences" in each instance refers to a portion of the total number of the particular identical sequences, and it does not refer to a portion of an individual particular identical sequence. In certain embodiments, "a substantial portion of the particular identical sequences" means at least 90% of the particular identical sequences. In certain embodiments, "a substantial portion of the particular identical sequences" means at least 95% of the particular identical sequences.

[077] In certain embodiments, the number of mismatches that may be present may vary in view of the complexity of the composition. Thus, in certain embodiments, fewer mismatches may be tolerated in a composition comprising

DNA from an entire genome than a composition in which fewer DNA sequences are present. For example, in certain embodiments, with a given number of mismatches, a probe may more likely hybridize to undesired sequences in a composition with the entire genomic DNA than in a composition with fewer DNA sequences, when the same hybridization conditions are employed for both compositions. Thus, that given number of mismatches may be appropriate for the composition with fewer DNA sequences, but fewer mismatches may be more optimal for the composition with the entire genomic DNA.

[078] In certain embodiments, sequences are complementary if they have no more than 20% mismatched nucleotides. In certain embodiments, sequences are complementary if they have no more than 15% mismatched nucleotides. In certain embodiments, sequences are complementary if they have no more than 10% mismatched nucleotides. In certain embodiments, sequences are complementary if they have no more than 5% mismatched nucleotides.

[079] In this application, a statement that one sequence hybridizes or binds to another sequence encompasses situations where the entirety of both of the sequences hybridize or bind to one another, and situations where only a portion of one or both of the sequences hybridizes or binds to the entire other sequence or to a portion of the other sequence. Here, the term "sequence" encompasses, but is not limited to, nucleic acid sequences, polynucleotides, oligonucleotides, probes, primers, primer-specific portions, target-specific portions, addressable portions, and oligonucleotide link elements.

[080] In certain embodiments, the term "to a measurably lesser extent" encompasses situations in which the event in question is reduced at least 10 fold.

In certain embodiments, the term "to a measurably lesser extent" encompasses situations in which the event in question is reduced at least 100 fold.

[081] In certain embodiments, a statement that a component may be, is, or has been "substantially removed" means that at least 90% of the component may be, is, or has been removed. In certain embodiments, a statement that a component may be, is, or has been "substantially removed" means that at least 95% of the component may be, is, or has been removed.

B. Certain Components

[082] In certain embodiments, target nucleic acid sequences may include RNA and DNA. Exemplary RNA target sequences include, but are not limited to, mRNA, rRNA, tRNA, viral RNA, and variants of RNA, such as splicing variants. Exemplary DNA target sequences include, but are not limited to, genomic DNA, plasmid DNA, phage DNA, nucleolar DNA, mitochondrial DNA, and chloroplast DNA.

[083] In certain embodiments, target nucleic acid sequences include, but are not limited to, cDNA, yeast artificial chromosomes (YAC's), bacterial artificial chromosomes (BAC's), other extrachromosomal DNA, and nucleic acid analogs. Exemplary nucleic acid analogs include, but are not limited to, LNAs, PNAs, PPG's, and other nucleic acid analogs.

[084] A variety of methods are available for obtaining a target nucleic acid sequence for use with the compositions and methods of the present invention. When the nucleic acid target is obtained through isolation from a biological matrix, certain isolation techniques include, but are not limited to, (1) organic extraction followed by ethanol precipitation, e.g., using a phenol/chloroform organic reagent

(e.g., Ausubel *et al.*, eds., *Current Protocols in Molecular Biology Volume 1*, Chapter 2, Section I, John Wiley & Sons, New York (1993)), in certain embodiments, using an automated DNA extractor, e.g., the Model 341 DNA Extractor available from Applied Biosystems (Foster City, CA); (2) stationary phase adsorption methods (e.g., Boom *et al.*, U.S. Patent No. 5,234,809; Walsh *et al.*, *Biotechniques* 10(4): 506-513 (1991)); and (3) salt-induced DNA precipitation methods (e.g., Miller *et al.*, *Nucleic Acids Research*,16(3): 9-10 (1988)), such precipitation methods being typically referred to as "salting-out" methods. In certain embodiments, the above isolation methods may be preceded by an enzyme digestion step to help eliminate unwanted protein from the sample, e.g., digestion with proteinase K, or other like proteases. *See, e.g.,* U.S. Patent Application Serial No. 09/724,613.

[085] In certain embodiments, a target nucleic acid sequence may be derived from any living, or once living, organism, including but not limited to prokaryote, eukaryote, plant, animal, and virus. In certain embodiments, the target nucleic acid sequence may originate from a nucleus of a cell, e.g., genomic DNA, or may be extranuclear nucleic acid, e.g., plasmid, mitrochondrial nucleic acid, various RNAs, and the like. In certain embodiments, if the sequence from the organism is RNA, it may be reverse-transcribed into a cDNA target nucleic acid sequence. Furthermore, in certain embodiments, the target nucleic acid sequence may be present in a double stranded or single stranded form.

[086] Exemplary target nucleic acid sequences include, but are not limited to, amplification products, ligation products, transcription products, reverse transcription products, primer extension products, methylated DNA, and cleavage

products. Exemplary amplification products include, but are not limited to, PCR and isothermal products.

[087] In certain embodiments, nucleic acids in a sample may be subjected to a cleavage procedure. In certain embodiments, such cleavage products may be targets.

[088] Different target nucleic acid sequences may be different portions of a single contiguous nucleic acid or may be on different nucleic acids. Different portions of a single contiguous nucleic acid may or may not overlap.

[089] In certain embodiments, a target nucleic acid sequence comprises an upstream or 5' region, a downstream or 3' region, and a "pivotal nucleotide" located in the upstream region or the downstream region (see, e.g., Figure 4). In certain embodiments, the pivotal nucleotide may be the nucleotide being detected by the probe set and may represent, for example, without limitation, a single polymorphic nucleotide in a multiallelic target locus. In certain embodiments, more than one pivotal nucleotide is present. In certain embodiments, one or more pivotal nucleotides is located in the upstream region, and one or more pivotal nucleotide is located in the downstream region. In certain embodiments, more than one pivotal nucleotide is located in the upstream region or the downstream region.

[090] The person of ordinary skill will appreciate that while a target nucleic acid sequence is typically described as a single-stranded molecule, the opposing strand of a double-stranded molecule comprises a complementary sequence that may also be used as a target sequence.

[091] A ligation probe set, according to certain embodiments, comprises two or more probes that comprise a target-specific portion that is designed to hybridize in a sequence-specific manner with a complementary region on a specific

target nucleic acid sequence (*see, e.g.*, probes 2 and 3 in Fig. 2). A probe of a ligation probe set may further comprise a primer-specific portion, an addressable portion, or a combination of these additional components. In certain embodiments, any of the probe's components may overlap any other probe component(s). For example, but without limitation, the target-specific portion may overlap the primer-specific portion. Also, without limitation, the addressable portion may overlap with the target-specific portion or the primer specific-portion, or both.

[092] In certain embodiments, at least one probe of a ligation probe set comprises the addressable portion located between the target-specific portion and the primer-specific portion (*see, e.g.*, probe 23 in Fig. 3). In certain embodiments, the probe's addressable portion may comprise a sequence that is the same as, or is complementary to, at least a portion of a labeled probe. In certain embodiments, the probe's primer-specific portion may comprise a sequence that is the same as, or is complementary to, at least a portion of a labeled probe. In certain embodiments, the probe's addressable portion is not complementary with target sequences, primer sequences, or probe sequences other than complementary portions of labeled probes.

[093] The sequence-specific portions of probes are of sufficient length to permit specific annealing to complementary sequences in primers, addressable portions, and targets as appropriate. In certain embodiments, the length of the addressable portions and target-specific portion are any number of nucleotides from 6 to 35. Detailed descriptions of probe design that provide for sequence-specific annealing can be found, among other places, in Diffenbach and Dveksler, PCR Primer, A Laboratory Manual, Cold Spring Harbor Press, 1995, and Kwok et al., Nucl. Acid Res. 18:999-1005 (1990).

[094] A ligation probe set according to certain embodiments comprises at least one first probe and at least one second probe that adjacently hybridize to the same target nucleic acid sequence. According to certain embodiments, a ligation probe set is designed so that the target-specific portion of the first probe will hybridize with the downstream target region (*see, e.g.*, probe 2 in Fig. 2) and the target-specific portion of the second probe will hybridize with the upstream target region (*see, e.g.*, probe 3 in Fig. 2). The sequence-specific portions of the probes are of sufficient length to permit specific annealing with complementary sequences in targets and primers, as appropriate. In certain embodiments, one of the at least one first probe and the at least one second probe in a probe set further comprises an addressable portion.

[095] Under appropriate conditions, adjacently hybridized probes may be ligated together to form a ligation product, provided that they comprise appropriate reactive groups, for example, without limitation, a free 3'-hydroxyl and 5'-phosphate group.

[096] According to certain embodiments, some ligation probe sets may comprise more than one first probe or more than one second probe to allow sequence discrimination between target sequences that differ by one or more nucleotides (see, e.g., Figure 5).

[097] According to certain embodiments of the invention, a ligation probe set is designed so that the target-specific portion of the first probe will hybridize with the downstream target region (see, e.g., the first probe in Fig. 4) and the target-specific portion of the second probe will hybridize with the upstream target region (see, e.g., the second probe in Fig. 4). In certain embodiments, a nucleotide base complementary to the pivotal nucleotide, the "pivotal complement" or "pivotal

complement nucleotide," is present on the proximal end of the second probe of the target-specific probe set (see, e.g., 5' end (PC) of the second probe in Fig. 4). In certain embodiments, the first probe may comprise the pivotal complement and addressable portion rather than the second probe (see, e.g., Fig. 5). The skilled artisan will appreciate that, in various embodiments, the pivotal nucleotide(s) may be located anywhere in the target sequence and that likewise, the pivotal complement(s) may be located anywhere within the target-specific portion of the probe(s). For example, according to various embodiments, the pivotal complement may be located at the 3' end of a probe, at the 5' end of a probe, or anywhere between the 3' end and the 5' end of a probe.

[098] In certain embodiments, when the first and second probes of the ligation probe set are hybridized to the appropriate upstream and downstream target regions, and when the pivotal complement is at the 5' end of one probe or the 3' end of the other probe, and the pivotal complement is base-paired with the pivotal nucleotide on the target sequence, the hybridized first and second probes may be ligated together to form a ligation product (see, e.g., Figure 5(2)-(3)). In the example shown in Figure 5(2)-(3), a mismatched base at the pivotal nucleotide, however, interferes with ligation, even if both probes are otherwise fully hybridized to their respective target regions.

[099] In certain embodiments, other mechanisms may be employed to avoid ligation of probes that do not include the correct complementary nucleotide at the pivotal complement. For example, in certain embodiments, conditions may be employed such that a probe of a ligation probe set will hybridize to the target sequence to a measurably lesser extent if there is a mismatch at the pivotal

nucleotide. Thus, in such embodiments, such non-hybridized probes will not be ligated to the other probe in the probe set.

[0100] In certain embodiments, the first probes and second probes in a ligation probe set are designed with similar melting temperatures (T_m). Where a probe includes a pivotal complement, in certain embodiments, the T_m for the probe(s) comprising the pivotal complement(s) of the target pivotal nucleotide sought will be approximately 4-15° C lower than the other probe(s) that do not contain the pivotal complement in the probe set. In certain such embodiments, the probe comprising the pivotal complement(s) will also be designed with a T_m near the ligation temperature. Thus, a probe with a mismatched nucleotide will more readily dissociate from the target at the ligation temperature. The ligation temperature, therefore, in certain embodiments provides another way to discriminate between, for example, multiple potential alleles in the target.

[0101] Further, in certain embodiments, ligation probe sets do not comprise a pivotal complement at the terminus of the first or the second probe (e.g., at the 3' end or the 5' end of the first or second probe). Rather, the pivotal complement is located somewhere between the 5' end and the 3' end of the first or second probe. In certain such embodiments, probes with target-specific portions that are fully complementary with their respective target regions will hybridize under high stringency conditions. Probes with one or more mismatched bases in the target-specific portion, by contrast, will hybridize to their respective target region to a measurably lesser extent. Both the first probe and the second probe must be hybridized to the target for a ligation product to be generated.

[0102] In certain embodiments, highly related sequences that differ by as little as a single nucleotide can be distinguished. For example, according to certain

embodiments, one can distinguish the two potential alleles in a biallelic locus as follows. One can combine a ligation probe set comprising two first probes, differing in their addressable portions and their pivotal complement (see, e.g., probes A and B in Fig. 5(2)), one second probe (see, e.g., probe Z in Fig. 5(1)), and the sample containing the target. All three probes will hybridize with the target sequence under appropriate conditions (see, e.g., Fig. 5(2)). Only the first probe with the hybridized pivotal complement, however, will be ligated with the hybridized second probe (see, e.g., Fig. 5(3)). Thus, if only one allele is present in the sample, only one ligation product for that target will be generated (see, e.g., ligation product A-Z in Fig. 5(4)). Both ligation products would be formed in a sample from a heterozygous individual. In certain embodiments, ligation of probes with a pivotal complement that is not complementary to the pivotal nucleotide may occur, but such ligation occurs to a measurably lesser extent than ligation of probes with a pivotal complement that is complementary to the pivotal nucleotide.

[0103] Many different signal moieties may be used in various embodiments of the present invention. For example, signal moieties include, but are not limited to, fluorophores, radioisotopes, chromogens, enzymes, antigens, heavy metals, dyes, phosphorescence groups, chemiluminescent groups, and electrochemical detection moieties. Exemplary fluorophores that may be used as signal moieties include, but are not limited to, rhodamine, cyanine 3 (Cy 3), cyanine 5 (Cy 5), fluorescein, Vic™, Liz™, Tamra™, 5-Fam™, 6-Fam™, and Texas Red (Molecular Probes). (Vic™, Liz™, Tamra™, 5-Fam™, and 6-Fam™ (all available from Applied Biosystems, Foster City, CA.) Exemplary radioisotopes include, but are not limited to, ^{32}P, ^{33}P, and ^{35}S. Signal moieties also include elements of multi-element indirect reporter systems, e.g., biotin/avidin, antibody/antigen, ligand/receptor,

enzyme/substrate, and the like, in which the element interacts with other elements of the system in order to effect a detectable signal. Certain exemplary multi-element systems include a biotin reporter group attached to a probe and an avidin conjugated with a fluorescent label. Detailed protocols for methods of attaching signal moieties to oligonucleotides can be found in, among other places, G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, CA (1996) and S.L. Beaucage et al., Current Protocols in Nucleic Acid Chemistry, John Wiley & Sons, New York, NY (2000).

[0104] As discussed above, the term "interaction probe" refers to a probe that comprises at least two moieties that can interact with one another to provide a detectably different signal value depending upon whether a given nucleic acid sequence is present or absent. In certain embodiments, one of the moieties is a signal moiety and the other moiety is a quencher moiety. The signal value that is detected from the signal moiety is different depending on whether the quencher moiety is sufficiently close to the signal moiety or is spaced apart from the signal moiety. In certain embodiments, the quencher moiety decreases the detectable signal value from the signal moiety when the quencher moiety is sufficiently close to the signal moiety. In certain embodiments, the quencher moiety decreases the detectable signal value to zero or close to zero when the quencher moiety is sufficiently close to the signal moiety.

[0105] In certain embodiments, one of the moieties of the interaction probe is a signal moiety and the other moiety is a donor moiety. The signal value that is detected from the signal moiety is different depending on whether the donor moiety is sufficiently close to the signal moiety or is spaced apart from the signal moiety. In certain embodiments, the donor moiety increases the detectable signal value

from the signal moiety when the donor moiety is sufficiently close to the signal moiety. In certain embodiments, the detectable signal value is zero or close to zero when the donor moiety is not sufficiently close to the signal moiety.

[0106] In certain embodiments employing a donor moiety and signal moiety, one may use certain energy-transfer fluorescent dyes. Certain nonlimiting exemplary pairs of donors (donor moieties) and acceptors (signal moieties) are illustrated, e.g., in U.S. Patent Nos. 5,863,727; 5,800,996; and 5,945,526. Use of certain such combinations of a donor and an acceptor have also been called FRET (Fluorescent Resonance Energy Transfer).

[0107] In certain embodiments, the moieties of the interaction probe are linked to one another by a link element such as, but not limited to, an oligonucleotide. In certain such embodiments, the presence of a sequence that hybridizes to a interaction probe impacts the proximity of the moieties to one another during the methods described herein. In various embodiments, the moieties may be attached to the link element in various ways known in the art. For example, certain nonlimiting protocols for attaching moieties to oligonucleotides are found in, among other places, G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, CA (1996) and S.L. Beaucage et al., Current Protocols in Nucleic Acid Chemistry, John Wiley & Sons, New York, NY (2000). In certain embodiments, an interaction probe comprises more than one signal moiety. In certain embodiments, an interaction probe comprises more than one quencher moiety. In certain embodiments, an interaction probe comprises more than one donor moiety.

[0108] According to certain embodiments, the interaction probe may be a "5'-nuclease probe," which comprises a signal moiety linked to a quencher moiety or a

donor moiety through a short oligonucleotide link element. When the 5'-nuclease probe is intact, the quencher moiety or the donor moiety influences the detectable signal from the signal moiety. According to certain embodiments, the 5'-nuclease probe binds to a specific nucleic acid sequence, and is cleaved by the 5' nuclease activity of at least one of a polymerase and another enzymatic construct when the probe is replaced by a newly polymerized strand during an amplification reaction such as PCR or some other strand displacement protocol.

[0109] When the oligonucleotide link element of the 5'-nuclease probe is cleaved, the detectable signal from the signal moiety changes when the signal moiety becomes further separated from the quencher moiety or the donor moiety. In certain such embodiments that employ a quencher moiety, the signal value increases when the signal moiety becomes further separated from the quencher moiety. In certain such embodiments that employ a donor moiety, the signal value decreases when the signal moiety becomes further separated from the donor moiety.

[0110] An example of a 5' nuclease probe according to certain embodiments is depicted in Figure 1A, where the labeled probe (LBP) includes a quencher moiety (Q) and a signal moiety (S). The nucleic acid sequence with which the interaction probe interacts in Figure 1A includes a 5' primer-specific portion P-SP1, an addressable portion (ASP), and a 3' primer-specific portion (P-SP2). The signal that is detected from the labeled probe increases with cleavage.

[0111] In certain embodiments, the 5'-nuclease probe is a 5'-nuclease fluorescent probe, in which the signal moiety is a fluorescent moiety and the quencher moiety is a fluorescence quencher moiety. When the probe is cleaved during a strand displacement protocol, the fluorescent moiety emits a detectable

fluorescent signal. In certain embodiments, a 5'-nuclease fluorescent probe may emit a given level of signal when it is hybridized to a complementary sequence prior to cleavage, and the level of the signal is increased with cleavage. Certain exemplary embodiments of 5'-nuclease fluorescent probes are described, e.g., in U.S. Patent No. 5,538,848, and exemplified by the TaqMan® probe molecule, which is part of the TaqMan® assay system (available from Applied Biosystems, Foster City, CA).

[0112] According to certain embodiments, the interaction probe may be a "hybridization dependent probe," which comprises a signal moiety linked to a quencher moiety or a donor moiety through an oligonucleotide link element. When the hybridization dependent probe is not bound to a given nucleic acid sequence, and is thus single stranded, the oligonucleotide link element can bend flexibly, and the quencher moiety or the donor moiety is sufficiently close to the signal moiety to influence the detectable signal from the signal moiety. In certain embodiments, the oligonucleotide link element of a hybridization dependent probe is designed such that when it is not hybridized to a given nucleic acid sequence, it folds back and hybridizes to itself (see, e.g., Figure 1C), e.g., a molecular beacon probe. See, e.g., U.S. Patent Nos. 5,118,801; 5,312,728; and 5,925,517. In certain embodiments, the oligonucleotide link element of a hybridization dependent probe does not hybridize to itself when it is not hybridized to the given nucleic acid sequence (see, e.g., Figure 1B).

[0113] When a hybridization dependent probe is bound to a given nucleic acid as double stranded nucleic acid, the quencher moiety or the donor moiety is spaced apart from the signal moiety such that the detectable signal is changed. In certain such embodiments that employ a quencher moiety, the signal value

increases when the signal moiety becomes further separated from the quencher moiety. In certain such embodiments that employ a donor moiety, the signal value decreases when the signal moiety becomes further separated from the donor moiety.

[0114] Examples of certain hybridization dependent probes according to certain embodiments are depicted in Figures 1B and 1C, where the labeled probe (LBP) includes a quencher moiety (Q) and a signal moiety (S). The nucleic acid sequence with which the interaction probe interacts in Figures 1B and 1C includes a 5' primer-specific portion P-SP1, an addressable portion (ASP), and a 3' primer-specific portion (P-SP2).

[0115] In certain embodiments of hybridization dependent probes, the signal moiety is a fluorescent moiety and the quencher moiety is a fluorescence quencher moiety. When the probe is hybridized to a specific nucleic acid sequence, the fluorescent moiety emits a detectable fluorescent signal. When the probe is not hybridized to a nucleic acid sequence and is intact, quenching occurs and little or no fluorescence is detected.

[0116] Certain exemplary embodiments of hybridization dependent probes are described, e.g., in U.S. Patent No. 5,723,591.

[0117] In certain embodiments, one employs nucleic acids in the hybridization dependent probes such that a substantial portion of the hybridization dependent probes are not cleaved by an enzyme during an amplification reaction. A "substantial portion of the hybridization dependent probes are not cleaved" refers to a portion of the total number of hybridization dependent probes that are designed to hybridize to a given nucleic sequence that is being amplified, and it does not refer to a portion of an individual probe. In certain embodiments, "a substantial

portion of hybridization dependent probes that are not cleaved" means that at least 90% of the hybridization dependent probes are not cleaved. In certain embodiments, at least 95% of the hybridization dependent probes are not cleaved. In certain embodiments, one employs PNA for some or all of the nucleic acids of a hybridization dependent probe.

[0118] In certain embodiments, one employs hybridization dependent probes in which a substantial portion of the hybridization dependent probes do not hybridize to an addressable portion or a complement of the addressable portion during an extension reaction. A "substantial portion of the hybridization dependent probes do not hybridize" here refers to a portion of the total number of hybridization dependent probes that are designed to hybridize to a given nucleic sequence that is being amplified, and it does not refer to a portion of an individual probe. In certain embodiments, "a substantial portion of hybridization dependent probes that do not hybridize" means that at least 90% of the hybridization dependent probes do not hybridize. In certain embodiments, at least 95% of the hybridization dependent probes do not hybridize.

[0119] According to certain embodiments, the interaction probe may be a "cleavable RNA probe," which comprises a signal moiety linked to a quencher moiety or a donor moiety through a short RNA link element. When the cleavable RNA probe is intact, the quencher moiety or the donor moiety influences the detectable signal from the signal moiety. According to certain embodiments, the cleavable RNA probe binds to a specific DNA sequence, and is cleaved by RNase H, or an agent with similar activity.

[0120] When the RNA link element of the cleavable RNA probe is cleaved, the detectable signal from the signal moiety changes when the signal moiety

becomes further separated from the quencher moiety or the donor moiety. In certain such embodiments that employ a quencher moiety, the signal value increases when the signal moiety becomes further separated from the quencher moiety. In certain such embodiments that employ a donor moiety, the signal value decreases when the signal moiety becomes further separated from the donor moiety.

[0121] In certain embodiments, if a particular nucleic acid sequence that is to be detected is present in a sample, a nucleic acid amplification procedure results in more DNA comprising the specific DNA sequence to which a cleavable RNA probe binds than if the particular nucleic acid sequence is not present in the sample. In such embodiments, one may determine the presence of the particular nucleic acid in the sample in view of the signal generated from the cleavable RNA probe during and/or after the amplification procedure. In certain embodiments, one may quantitate the amount of a particular nucleic acid in a sample in view of the signal generated from a cleavable RNA probe during and/or after the amplification procedure.

[0122] In certain embodiments, the cleavable RNA probe is a cleavable RNA fluorescent probe, in which the signal moiety is a fluorescent moiety and the quencher moiety is a fluorescence quencher moiety. When the probe is cleaved, the fluorescent moiety emits a detectable fluorescent signal. In certain embodiments, a cleavable RNA probe may emit a given level of signal when it is hybridized to a complementary sequence prior to cleavage, and the level of the signal is increased with cleavage.

[0123] According to certain embodiments, the interaction probe may be a "structure-specific nuclease probe," which comprises a signal moiety linked to a

quencher moiety or a donor moiety through a short oligonucleotide link element. When the structure-specific nuclease probe is intact, the quencher moiety or the donor moiety influences the detectable signal from the signal moiety. According to certain embodiments, the structure-specific nuclease probe binds to a specific nucleic acid sequence, and is cleaved by a structure-specific nuclease if it is appropriately hybridized to the specific nucleic acid sequence.

[0124] When the oligonucleotide link element of the structure-specific nuclease probe is cleaved, the detectable signal from the signal moiety changes when the signal moiety becomes further separated from the quencher moiety or the donor moiety. In certain such embodiments that employ a quencher moiety, the signal value increases when the signal moiety becomes further separated from the quencher moiety. In certain such embodiments that employ a donor moiety, the signal value decreases when the signal moiety becomes further separated from the donor moiety.

[0125] In certain embodiments, the structure-specific nuclease probe is a structure-specific nuclease fluorescent probe, in which the signal moiety is a fluorescent moiety and the quencher moiety is a fluorescence quencher moiety. When the probe is cleaved, the fluorescent moiety emits a detectable fluorescent signal. In certain embodiments, a structure-specific nuclease probe may emit a given level of signal when it is hybridized to a complementary sequence prior to cleavage, and the level of the signal is increased with cleavage.

[0126] In certain embodiments, one employs a structure-specific nuclease probe comprising a flap that does not substantially hybridize to the addressable portion and employs a flap endonuclease (FEN) as the structure-specific nuclease. An exemplary embodiment is shown in Figure 8. The structure-specific nuclease

probe in Figure 8 includes a flap portion that does not hybridize to the addressable portion, a hybridizing portion that hybridizes to the addressable portion, and a FEN cleavage position nucleotide between the flap portion and the hybridizing portion. The FEN cleavage position nucleotide is designed to be complementary to the nucleotide of the addressable portion that is immediately 3' to the nucleotide that hybridizes to the 5' end nucleotide of the probe's hybridizing portion. The flap portion includes a signal moiety attached to it and the hybridizing portion includes a quencher moiety or a donor moiety attached to it.

[0127] As shown in the embodiments depicted in Figure 8, another oligonucleotide X is designed to hybridize to the addressable portion 3' to the portion of the addressable portion that hybridizes to the hybridizing portion of the structure-specific nuclease probe. If the appropriate addressable portion is present, FEN will cleave the structure-specific nuclease probe such that the signal moiety becomes separated from the quenching moiety or donor moiety.

[0128] According to certain embodiments, the interaction probe may comprise two oligonucleotides that hybridize to a given nucleic acid sequence adjacent to one another. In certain embodiments, one of the oligonucleotides comprises a signal moiety and one of the oligonucleotides comprises a quencher moiety or a donor moiety. When both oligonucleotides are hybridized to the given nucleic acid sequence, the quencher moiety or the donor moiety is sufficiently close to the signal moiety to influence the detectable signal from the signal moiety.

[0129] In certain such embodiments that employ a donor moiety, the signal value increases when the two oligonucleotides are hybridized to the given nucleic acid sequence. In certain such embodiments that employ a quencher moiety, the signal value decreases when the two oligonucleotides are hybridized to the given

nucleic acid sequence. In certain embodiments, the signal moiety is a fluorescent moiety.

[0130] Other examples of suitable labeled probes according to certain embodiments are i-probes, scorpion probes, eclipse probes, and others. Exemplary, but nonlimiting, probes are discussed, for example, in Whitcombe et al., Nat. Biotechnol., 17(8):804-807 (1999) (includes scorpion probes); Thelwell et al., Nucleic Acids Res., 28(19):3752-3761 (2000) (includes scorpion probes); Afonina et al., Biotechniques, 32(4): (2002) (includes eclipse probes); Li et al., "A new class of homogeneous nucleic acid probes based on specific displacement hybridization", Nucleic Acids Res., 30(2):E5 (2002); Kandimall et al., Bioorg. Med. Chem., 8(8):1911-1916 (2000); Isacsson et al., Mol. Cell. Probes, 14(5):321-328 (2000); French et al, Mol. Cell. Probes, 15(6):363-374 (2001); and Nurmi et al., "A new label technology for the detection of specific polymerase chain reaction products in a closed tube", Nucleic Acids Res., 28(8), E28 (2000). Exemplary quencher moieties according to certain embodiments may be those available from Epoch Biosciences, Bothell, Washington.

[0131] In certain embodiments, one may use a labeled probe and a threshold difference between first and second detectable signal values to detect the presence or absence of a target nucleic acid in a sample. In such embodiments, if the difference between the first and second detectable signal values is the same as or greater than the threshold difference, i.e., there is a threshold difference, one concludes that the target nucleic acid is present. If the difference between the first and second detectable signal values is less than the threshold difference, i.e., there is no threshold difference, one concludes that the target nucleic acid is absent.

[0132] Certain nonlimiting examples of how one may set a threshold difference according to certain embodiments follow.

[0133] First, in certain embodiments, a labeled probe that is not hybridized to a complementary sequence may have a first detectable signal value of zero. In certain embodiments, when one forms an amplification reaction composition comprising the labeled probe, and any unligated ligation probes and ligation products that include complementary addressable portions, before amplification, the detectable signal value may increase to 0.4. In certain such embodiments, when such an amplification reaction composition does not include any ligation products comprising the complementary addressable portion, the detectable signal value may remain at 0.4 during and/or after an amplification reaction. (In other words, the second detectable signal value is 0.4.) In certain such embodiments, when such an amplification reaction composition, however, includes a ligation product comprising a complementary addressable portion, the detectable signal value may increase to 2 during and/or after an amplification reaction. (In other words, the second detectable signal value is 2.)

[0134] Thus, in certain such embodiments, one may set a threshold difference between first and second detectable signal values at a value somewhere between a value just above 0.4 to about 2. For example, one may set the threshold difference at somewhere between 0.5 to 2.

[0135] Second, in certain embodiments, a labeled probe that is not hybridized to a complementary sequence may have an first detectable signal value of zero. In certain embodiments, when one forms an amplification reaction composition comprising the labeled probe, and any unligated ligation probes and ligation products that include complementary addressable portions, before

amplification, the detectable signal value may increase to 0.4. In certain such embodiments, when such an amplification reaction composition does not include any ligation products comprising the complementary addressable portion, the detectable signal value may increase to 0.7 during and/or after an amplification reaction. (In other words, the second detectable signal value is 0.7.) In certain such embodiments, when such an amplification reaction composition, however, includes a ligation product comprising a complementary addressable portion, the detectable signal value may increase to 2 during and/or after an amplification reaction. (In other words, the second detectable signal value is 2.)

[0136] Thus, in certain such embodiments, one may set a threshold difference between first and second detectable signal values at a value somewhere between a value just above 0.7 to about 2. For example, one may set the threshold difference at somewhere between 0.8 to 2.

[0137] Third, in certain embodiments, a labeled probe that is not hybridized to a complementary sequence may have a first detectable signal value of zero. In certain embodiments, when one forms an amplification reaction composition comprising the labeled probe, and any unligated ligation probes and ligation products that include complementary addressable portions, before amplification, the detectable signal value may increase to 0.4. In certain such embodiments, when such an amplification reaction composition does not include any ligation products comprising the complementary addressable portion, the detectable signal value may increase linearly during and/or after an amplification reaction. (In other words, the second detectable signal value is linearly increased from the first detectable signal value.) In certain such embodiments, when such an amplification reaction composition, however, includes a ligation product comprising a

complementary addressable portion, the detectable signal value may increase exponentially during and/or after an amplification reaction. (In other words, the second detectable signal value is exponentially increased from the first detectable signal value.)

[0138] Thus, in certain such embodiments, one may measure detectable signal values at two or more points during amplification, and at the end of the amplification reaction, to determine if the increase in detectable signal value is linear or exponential. In certain embodiments, one may measure detectable signal values at three or more points during amplification to determine if the increase in detectable signal value is linear or exponential. In certain embodiments, if the increase is exponential, there is a threshold difference between the first and second detectable signal values.

[0139] In certain embodiments, one may employ different labeled probes that are specific to different addressable portions. In certain such embodiments, one may employ different labeled probes that comprise different sequences and detectably different signal moieties. Detectably different signal moieties include, but are not limited to, moieties that emit light of different wavelengths, moieties that absorb light of different wavelengths, moieties that have different fluorescent decay lifetimes, moieties that have different spectral signatures, and moieties that have different radioactive decay properties.

[0140] According to certain embodiments, a minor groove binder may be attached to at least one labeled probe. Certain exemplary minor groove binders and certain exemplary methods of attaching minor groove binders to oligonucleotides are discussed, e.g., in U.S. Patent Nos. 5,801,155 and 6,084,102.

Certain exemplary minor groove binders are those that are available from Epoch Biosciences, Bothell, Washington.

[0141] A primer set according to certain embodiments comprises at least one primer capable of hybridizing with the primer-specific portion of at least one probe of a ligation probe set. In certain embodiments, a primer set comprises at least one first primer and at least one second primer, wherein the at least one first primer specifically hybridizes with one probe of a ligation probe set (or a complement of such a probe) and the at least one second primer of the primer set specifically hybridizes with a second probe of the same ligation probe set (or a complement of such a probe). In certain embodiments, the first and second primers of a primer set have different hybridization temperatures, to permit temperature-based asymmetric PCR reactions.

[0142] The skilled artisan will appreciate that while the probes and primers of the invention may be described in the singular form, a plurality of probes or primers may be encompassed by the singular term, as will be apparent from the context. Thus, for example, in certain embodiments, a ligation probe set typically comprises a plurality of first probes and a plurality of second probes.

[0143] The criteria for designing sequence-specific primers and probes are well known to persons of ordinary skill in the art. Detailed descriptions of primer design that provide for sequence-specific annealing can be found, among other places, in Diffenbach and Dveksler, PCR Primer, A Laboratory Manual, Cold Spring Harbor Press, 1995, and Kwok et al. (Nucl. Acid Res. 18:999-1005, 1990). The sequence-specific portions of the primers are of sufficient length to permit specific annealing to complementary sequences in ligation products and amplification products, as appropriate.

[0144] According to certain embodiments, a primer set of the present invention comprises at least one second primer. In certain embodiments, the second primer in that primer set is designed to hybridize with a 3' primer-specific portion of a ligation or amplification product in a sequence-specific manner (*see, e.g.,* Figure 2C). In certain embodiments, the primer set further comprises at least one first primer. In certain embodiments, the first primer of a primer set is designed to hybridize with the complement of the 5' primer-specific portion of that same ligation or amplification product in a sequence-specific manner.

[0145] A universal primer or primer set may be employed according to certain embodiments. In certain embodiments, a universal primer or a universal primer set hybridizes with two or more of the probes, ligation products, or amplification products in a reaction, as appropriate. When universal primer sets are used in certain amplification reactions, such as, but not limited to, PCR, qualitative or quantitative results may be obtained for a broad range of template concentrations.

[0146] Certain embodiments include a ligation agent. For example, ligase is an enzymatic ligation agent that, under appropriate conditions, forms phosphodiester bonds between the 3'-OH and the 5'-phosphate of adjacent nucleotides in DNA or RNA molecules, or hybrids. Exemplary ligases include, but are not limited to, *Tth* K294R ligase and *Tsp* AK16D ligase. See, e.g., Luo et al., Nucleic Acids Res., 24(14):3071-3078 (1996); Tong et al., Nucleic Acids Res., 27(3):788-794 (1999); and Published PCT Application No. WO 00/26381. Temperature sensitive ligases, include, but are not limited to, T4 DNA ligase, T7 DNA ligase, and *E. coli* ligase. In certain embodiments, thermostable ligases include, but are not limited to, *Taq* ligase, *Tth* ligase, *Tsc* ligase, and *Pfu* ligase.

Certain thermostable ligases may be obtained from thermophilic or hyperthermophilic organisms, including but not limited to, prokaryotic, eucaryotic, or archael organisms. Certain RNA ligases may be employed in certain embodiments. In certain embodiments, the ligase is a RNA dependent DNA ligase, which may be employed with RNA template and DNA ligation probes. An exemplary, but nonlimiting example, of a ligase with such RNA dependent DNA ligase activity is T4 DNA ligase. In certain embodiments, the ligation agent is an "activating" or reducing agent.

[0147] Chemical ligation agents include, without limitation, activating, condensing, and reducing agents, such as carbodiimide, cyanogen bromide (BrCN), N-cyanoimidazole, imidazole, 1-methylimidazole/carbodiimide/ cystamine, dithiothreitol (DTT) and ultraviolet light. Autoligation, i.e., spontaneous ligation in the absence of a ligating agent, is also within the scope of certain embodiments of the invention. Detailed protocols for chemical ligation methods and descriptions of appropriate reactive groups can be found, among other places, in Xu et al., Nucleic Acid Res., 27:875-81 (1999); Gryaznov and Letsinger, Nucleic Acid Res. 21:1403-08 (1993); Gryaznov et al., Nucleic Acid Res. 22:2366-69 (1994); Kanaya and Yanagawa, Biochemistry 25:7423-30 (1986); Luebke and Dervan, Nucleic Acids Res. 20:3005-09 (1992); Sievers and von Kiedrowski, Nature 369:221-24 (1994); Liu and Taylor, Nucleic Acids Res. 26:3300-04 (1999); Wang and Kool, Nucleic Acids Res. 22:2326-33 (1994); Purmal et al., Nucleic Acids Res. 20:3713-19 (1992); Ashley and Kushlan, Biochemistry 30:2927-33 (1991); Chu and Orgel, Nucleic Acids Res. 16:3671-91 (1988); Sokolova et al., FEBS Letters 232:153-55 (1988); Naylor and Gilham, Biochemistry 5:2722-28 (1966); and U.S. Patent No. 5,476,930.

[0148] In certain embodiments, at least one polymerase is included. In certain embodiments, at least one thermostable polymerase is included. Exemplary thermostable polymerases, include, but are not limited to, *Taq* polymerase, *Pfx* polymerase, *Pfu* polymerase, Vent® polymerase, Deep Vent™ polymerase, *Pwo* polymerase, *Tth* polymerase, UlTma polymerase and enzymatically active mutants and variants thereof. Descriptions of these polymerases may be found, among other places, at the world wide web URL: the-scientist.com/yr1998/jan/profile 1_980105. html; at the world wide web URL: the-scientist.com/yr2001/jan/profile _010903. html; at the world wide web URL: the-scientist.com/yr2001/sep/profile2 _010903. html; at the article The Scientist 12(1):17 (Jan. 5, 1998); and at the article The Scientist 15(17):1 (Sep. 3, 2001).

[0149] The skilled artisan will appreciate that the complement of the disclosed probe, target, and primer sequences, or combinations thereof, may be employed in certain embodiments of the invention. For example, without limitation, a genomic DNA sample may comprise both the target sequence and its complement. Thus, in certain embodiments, when a genomic sample is denatured, both the target sequence and its complement are present in the sample as single-stranded sequences. In certain embodiments, ligation probes may be designed to specifically hybridize to an appropriate sequence, either the target sequence or its complement.

C. Certain Exemplary Component Methods

[0150] Ligation according to the present invention comprises any enzymatic or chemical process wherein an internucleotide linkage is formed between the opposing ends of nucleic acid sequences that are adjacently hybridized to a

template. Additionally, the opposing ends of the annealed nucleic acid sequences should be suitable for ligation (suitability for ligation is a function of the ligation method employed). The internucleotide linkage may include, but is not limited to, phosphodiester bond formation. Such bond formation may include, without limitation, those created enzymatically by a DNA or RNA ligase, such as bacteriophage T4 DNA ligase, T4 RNA ligase, T7 DNA ligase, *Thermus thermophilus* (*Tth*) ligase, *Thermus aquaticus* (*Taq*) ligase, or *Pyrococcus furiosus* (*Pfu)* ligase. Other internucleotide linkages include, without limitation, covalent bond formation between appropriate reactive groups such as between an α-haloacyl group and a phosphothioate group to form a thiophosphorylacetylamino group; and between a phosphorothioate and a tosylate or iodide group to form a 5'-phosphorothioester or pyrophosphate linkages.

[0151] In certain embodiments, chemical ligation may, under appropriate conditions, occur spontaneously such as by autoligation. Alternatively, in certain embodiments, "activating" or reducing agents may be used. Examples of activating agents and reducing agents include, without limitation, carbodiimide, cyanogen bromide (BrCN), imidazole, 1-methylimidazole/carbodiimide/cystamine, N-cyanoimidazole, dithiothreitol (DTT) and ultraviolet light. Nonenzymatic ligation according to certain embodiments may utilize specific reactive groups on the respective 3' and 5' ends of the aligned probes.

[0152] In certain embodiments, ligation generally comprises at least one cycle of ligation, for example, the sequential procedures of: hybridizing the target-specific portions of a first probe and a second probe, that are suitable for ligation, to their respective complementary regions on a target nucleic acid sequence; ligating the 3' end of the first probe with the 5' end of the second probe to form a ligation

product; and denaturing the nucleic acid duplex to separate the ligation product from the target nucleic acid sequence. The cycle may or may not be repeated. For example, without limitation, by thermocycling the ligation reaction to linearly increase the amount of ligation product.

[0153] According to certain embodiments, one may use ligation techniques such as gap-filling ligation, including, without limitation, gap-filling OLA and LCR, bridging oligonucleotide ligation, and correction ligation. Descriptions of these techniques can be found, among other places, in U.S. Patent Number 5,185,243, published European Patent Applications EP 320308 and EP 439182, and published PCT Patent Application WO 90/01069.

[0154] In certain embodiments, one forms a test composition for a subsequent amplification reaction by subjecting a ligation reaction composition to at least one cycle of ligation. In certain embodiments, after ligation, the test composition may be used directly in the subsequent amplification reaction. In certain embodiments, prior to the amplification reaction, the test composition may be subjected to a purification technique that results in a test composition that includes less than all of the components that may have been present after the at least one cycle of ligation. For example, in certain embodiments, one may purify the ligation product.

[0155] Purifying the ligation product according to certain embodiments comprises any process that removes at least some unligated probes, target nucleic acid sequences, enzymes, and/or accessory agents from the ligation reaction composition following at least one cycle of ligation. Such processes include, but are not limited to, molecular weight/size exclusion processes, e.g., gel filtration chromatography or dialysis, sequence-specific hybridization-based pullout

methods, affinity capture techniques, precipitation, adsorption, or other nucleic acid purification techniques. The skilled artisan will appreciate that purifying the ligation product prior to amplification in certain embodiments reduces the quantity of primers needed to amplify the ligation product, thus reducing the cost of detecting a target sequence. Also, in certain embodiments, purifying the ligation product prior to amplification may decrease possible side reactions during amplification and may reduce competition from unligated probes during hybridization.

[0156] Hybridization-based pullout (HBP) according to certain embodiments of the present invention comprises a process wherein a nucleotide sequence complementary to at least a portion of one probe (or its complement), for example, the primer-specific portion, is bound or immobilized to a solid or particulate pullout support (see, e.g., U.S. Patent No. 6,124,092). In certain embodiments, a composition comprising ligation product, target sequences, and unligated probes is exposed to the pullout support. The ligation product, under appropriate conditions, hybridizes with the support-bound sequences. The unbound components of the composition are removed, purifying the ligation products from those ligation reaction composition components that do not contain sequences complementary to the sequence on the pullout support. One subsequently removes the purified ligation products from the support and combines them with at least one primer set to form a first amplification reaction composition. The skilled artisan will appreciate that, in certain embodiments, additional cycles of HBP using different complementary sequences on the pullout support may remove all or substantially all of the unligated probes, further purifying the ligation product.

[0157] Amplification according to the present invention encompasses a broad range of techniques for amplifying nucleic acid sequences, either linearly or

exponentially. Exemplary amplification techniques include, but are not limited to, PCR or any other method employing a primer extension step, and transcription or any other method of generating at least one RNA transcription product. Other nonlimiting examples of amplification are ligase detection reaction (LDR), and ligase chain reaction (LCR). Amplification methods may comprise thermal-cycling or may be performed isothermally. The term "amplification product" includes products from any number of cycles of amplification reactions, primer extension reactions, and RNA transcription reactions, unless otherwise apparent from the context.

[0158] In certain embodiments, amplification methods comprise at least one cycle of amplification, for example, but not limited to, the sequential procedures of: hybridizing primers to primer-specific portions of the ligation product or amplification products from any number of cycles of an amplification reaction; synthesizing a strand of nucleotides in a template-dependent manner using a polymerase; and denaturing the newly-formed nucleic acid duplex to separate the strands. The cycle may or may not be repeated.

[0159] Descriptions of certain amplification techniques can be found, among other places, in H. Ehrlich et al., Science, 252:1643-50 (1991), M. Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press, New York, NY (1990), R. Favis et al., Nature Biotechnology 18:561-64 (2000), and H.F. Rabenau et al., Infection 28:97-102 (2000); Sambrook and Russell, Ausbel et al.

[0160] Primer extension according to the present invention is an amplification process comprising elongating a primer that is annealed to a template in the 5' to 3' direction using a template-dependent polymerase. According to certain embodiments, with appropriate buffers, salts, pH, temperature, and nucleotide

triphosphates, including analogs and derivatives thereof, a template dependent polymerase incorporates nucleotides complementary to the template strand starting at the 3'-end of an annealed primer, to generate a complementary strand. Detailed descriptions of primer extension according to certain embodiments can be found, among other places in Sambrook et al., Sambrook and Russell, and Ausbel et al.

[0161] Transcription according to certain embodiments is an amplification process comprising an RNA polymerase interacting with a promoter on a single- or double-stranded template and generating a RNA polymer in a 5' to 3' direction. In certain embodiments, the transcription reaction composition further comprises transcription factors. RNA polymerases, including but not limited to T3, T7, and SP6 polymerases, according to certain embodiments, can interact with double-stranded promoters. Detailed descriptions of transcription according to certain embodiments can be found, among other places in Sambrook et al., Sambrook and Russell, and Ausbel et al.

[0162] Certain embodiments of amplification may employ multiplex PCR, in which multiple target sequences are simultaneously amplified (*see, e.g.*, H. Geada et al., Forensic Sci. Int. 108:31-37 (2000) and D.G. Wang et al., Science 280:1077-82 (1998)).

[0163] In certain embodiments, one employs asymmetric PCR. According to certain embodiments, asymmetric PCR comprises an amplification reaction composition comprising (i) at least one primer set in which there is an excess of one primer (relative to the other primer in the primer set); (ii) at least one primer set that comprises only a first primer or only a second primer; (iii) at least one primer set that, during given amplification conditions, comprises a primer that results in amplification of one strand and comprises another primer that is disabled; or (iv) at

least one primer set that meets the description of both (i) and (iii) above. Consequently, when the ligation product is amplified, an excess of one strand of the amplification product (relative to its complement) is generated.

[0164] In certain embodiments, one may use at least one primer set wherein the melting temperature (Tm_{50}) of one of the primers is higher than the Tm_{50} of the other primer. Such embodiments have been called asynchronous PCR (A-PCR). See, e.g., U.S. Patent Application Serial No. 09/875,211, filed June 5, 2001. In certain embodiments, the Tm_{50} of the first primer is at least 4-15° C different from the Tm_{50} of the second primer. In certain embodiments, the Tm_{50} of the first primer is at least 8-15° C different from the Tm_{50} of the second primer. In certain embodiments, the Tm_{50} of the first primer is at least 10-15° C different from the Tm_{50} of the second primer. In certain embodiments, the Tm_{50} of the first primer is at least 10-12° C different from the Tm_{50} of the second primer. In certain embodiments, in at least one primer set, the Tm_{50} of the at least one first primer differs from the melting temperature of the at least one second primer by at least about 4° C, by at least about 8° C, by at least about 10° C, or by at least about 12° C.

[0165] In certain embodiments of A-PCR, in addition to the difference in Tm_{50} of the primers in a primer set, there is also an excess of one primer relative to the other primer in the primer set. In certain embodiments, there is a five to twenty-fold excess of one primer relative to the other primer in the primer set. In certain embodiments of A-PCR, the primer concentration is at least 50mM.

[0166] In A-PCR according to certain embodiments, one may use conventional PCR in the first cycles such that both primers anneal and both strands are amplified. By raising the temperature in subsequent cycles, however, one may

disable the primer with the lower Tm such that only one strand is amplified. Thus, the subsequent cycles of A-PCR in which the primer with the lower Tm is disabled result in asymmetric amplification. Consequently, when the ligation product is amplified, an excess of one strand of the amplification product (relative to its complement) is generated.

[0167] According to certain embodiments of A-PCR, the level of amplification can be controlled by changing the number of cycles during the first phase of conventional PCR cycling. In such embodiments, by changing the number of initial conventional cycles, one may vary the amount of the double strands that are subjected to the subsequent cycles of PCR at the higher temperature in which the primer with the lower Tm is disabled.

[0168] In certain embodiments, an A-PCR protocol may comprise use of a pair of primers, each of which has a concentration of at least 50mM. In certain embodiments, conventional PCR, in which both primers result in amplification, is performed for the first 20-30 cycles. In certain embodiments, after 20-30 cycles of conventional PCR, the annealing temperature increases to 66-70°C, and PCR is performed for 5 to 40 cycles at the higher annealing temperature. In such embodiments, the lower Tm primer is disabled during such 5 to 40 cycles at higher annealing temperature. In such embodiments, asymmetric amplification occurs during the second phase of PCR cycles at a higher annealing temperature.

[0169] In certain embodiments, one employs asymmetric reamplification. According to certain embodiments, asymmetric reamplification comprises generating single-stranded amplification product in a second amplification process. In certain embodiments, the double-stranded amplification product of a first amplification process serves as the amplification target in the asymmetric

reamplification process. In certain embodiments, one may achieve asymmetric reamplification using asynchronous PCR in which initial cycles of PCR conventionally amplify two strands and subsequent cycles are performed at a higher annealing temperature that disables one of the primers of a primer set as discussed above. In certain embodiments, the second amplification reaction composition comprises at least one primer set which comprises the at least one first primer, or the at least one second primer of a primer set, but typically not both. The skilled artisan understands that, in certain embodiments, asymmetric reamplification will also eventually occur if the primers in the primer set are not present in an equimolar ratio. In certain asymmetric reamplification methods, typically only single-stranded amplicons are generated since the second amplification reaction composition comprises only first or second primers from each primer set or a non-equimolar ratio of first and second primers from a primer set.

[0170] In certain embodiments, additional polymerase may also be a component of the second amplification reaction composition. In certain embodiments, there may be sufficient residual polymerase from the first amplification composition to synthesize the second amplification product.

[0171] Methods of optimizing amplification reactions are well known to those skilled in the art. For example, it is well known that PCR may be optimized by altering times and temperatures for annealing, polymerization, and denaturing, as well as changing the buffers, salts, and other reagents in the reaction composition. Optimization may also be affected by the design of the amplification primers used. For example, the length of the primers, as well as the G-C:A-T ratio may alter the efficiency of primer annealing, thus altering the amplification reaction. See James

G. Wetmur, "Nucleic Acid Hybrids, Formation and Structure," in Molecular Biology and Biotechnology, pp.605-8, (Robert A. Meyers ed., 1995).

[0172] To detect whether a particular sequence is present, in certain embodiments, a labeled probe is included in the amplification reaction. According to certain embodiments, the labeled probe indicates the presence or absence (or amount) of a specific nucleic acid sequence in the reaction. These include, but are not limited to, 5'-nuclease probes, cleavage RNA probes, structure-specific nuclease probes, and hybridization dependent probes. In certain embodiments, the labeled probe comprises a fluorescing dye connected to a quenching molecule through a link element, e.g., through a specific oligonucleotide. Examples of such systems are described, e.g., in U.S. Patent Nos. 5,538,848 and 5,723,591.

[0173] Other examples of suitable labeled probes according to certain embodiments are i-probes, scorpion probes, eclipse probes, and others. Exemplary, but nonlimiting, probes are discussed, for example, in Whitcombe et al., Nat. Biotechnol., 17(8):804-807 (1999) (includes scorpion probes); Thelwell et al., Nucleic Acids Res., 28(19):3752-3761 (2000) (includes scorpion probes); Afonina et al., Biotechniques, 32(4): (2002) (includes eclipse probes); Li et al., "A new class of homogeneous nucleic acid probes based on specific displacement hybridization", Nucleic Acids Res., 30(2):E5 (2002); Kandimall et al., Bioorg. Med. Chem., 8(8):1911-1916 (2000); Isacsson et al., Mol. Cell. Probes, 14(5):321-328 (2000); French et al, Mol. Cell. Probes, 15(6):363-374 (2001); and Nurmi et al., "A new label technology for the detection of specific polymerase chain reaction products in a closed tube", Nucleic Acids Res., 28(8), E28 (2000).

[0174] In certain embodiments, the amount of labeled probe that gives a fluorescent signal in response to an emitted light typically relates to the amount of

nucleic acid produced in the amplification reaction. Thus, in certain embodiments, the amount of fluorescent signal is related to the amount of product created in the amplification reaction. In such embodiments, one can therefore measure the amount of amplification product by measuring the intensity of the fluorescent signal from the fluorescent indicator. According to certain embodiments, one can employ an internal standard to quantify the amplification product indicated by the fluorescent signal. See, e.g., U.S. Patent No. 5,736,333.

[0175] Devices have been developed that can perform a thermal cycling reaction with compositions containing a fluorescent indicator, emit a light beam of a specified wavelength, read the intensity of the fluorescent dye, and display the intensity of fluorescence after each cycle. Devices comprising a thermal cycler, light beam emitter, and a fluorescent signal detector, have been described, e.g., in U.S. Patent Nos. 5,928,907; 6,015,674; and 6,174,670, and include, but are not limited to the ABI Prism® 7700 Sequence Detection System (Applied Biosystems, Foster City, California) and the ABI GeneAmp® 5700 Sequence Detection System (Applied Biosystems, Foster City, California).

[0176] In certain embodiments, each of these functions may be performed by separate devices. For example, if one employs a Q-beta replicase reaction for amplification, the reaction may not take place in a thermal cycler, but could include a light beam emitted at a specific wavelength, detection of the fluorescent signal, and calculation and display of the amount of amplification product.

[0177] In certain embodiments, combined thermal cycling and fluorescence detecting devices can be used for precise quantification of target nucleic acid sequences in samples. In certain embodiments, fluorescent signals can be detected and displayed during and/or after one or more thermal cycles, thus

permitting monitoring of amplification products as the reactions occur in "real time." In certain embodiments, one can use the amount of amplification product and number of amplification cycles to calculate how much of the target nucleic acid sequence was in the sample prior to amplification.

[0178] According to certain embodiments, one could simply monitor the amount of amplification product after a predetermined number of cycles sufficient to indicate the presence of the target nucleic acid sequence in the sample. One skilled in the art can easily determine, for any given sample type, primer sequence, and reaction condition, how many cycles are sufficient to determine the presence of a given target polynucleotide.

[0179] According to certain embodiments, the amplification products can be scored as positive or negative as soon as a given number of cycles is complete. In certain embodiments, the results may be transmitted electronically directly to a database and tabulated. Thus, in certain embodiments, large numbers of samples may be processed and analyzed with less time and labor required.

[0180] According to certain embodiments, different labeled probes may distinguish between different target nucleic acid sequences. A non-limiting example of such a probe is a 5'-nuclease fluorescent probe, such as a TaqMan® probe molecule, wherein a fluorescent molecule is attached to a fluorescence-quenching molecule through an oligonucleotide link element. In certain embodiments, the oligonucleotide link element of the 5'-nuclease fluorescent probe binds to a specific sequence of an addressable portion or its complement. In certain embodiments, different 5'-nuclease fluorescent probes, each fluorescing at different wavelengths, can distinguish between different amplification products within the same amplification reaction.

[0181] For example, in certain embodiments, one could use two different 5'-nuclease fluorescent probes that fluoresce at two different wavelengths (WL_A and WL_B) and that are specific to two different addressable portions of two different ligation products (A' and B', respectively). Ligation product A' is formed if target nucleic acid sequence A is in the sample, and ligation product B' is formed if target nucleic acid sequence B is in the sample. In certain embodiments, ligation product A' and/or B' may form even if the appropriate target nucleic acid sequence is not in the sample, but such ligation occurs to a measurably lesser extent than when the appropriate target nucleic acid sequence is in the sample. After amplification, one can determine which specific target nucleic acid sequences are present in the sample based on the wavelength of signal detected. Thus, if an appropriate detectable signal value of only wavelength WL_A is detected, one would know that the sample includes target nucleic acid sequence A, but not target nucleic acid sequence B. If an appropriate detectable signal value of both wavelengths WL_A and WL_B are detected, one would know that the sample includes both target nucleic acid sequence A and target nucleic acid sequence B.

D. Certain Exemplary Embodiments of Detecting Targets

[0182] The present invention is directed to methods, reagents, and kits for detecting the presence or absence of (or quantitating) target nucleic acid sequences in a sample, using ligation and amplification reactions. When a particular target nucleic acid sequence is present in a sample, a ligation product is formed that includes an addressable portion. Labeled probes are employed that provide a different detectable signal value depending upon whether a complementary sequence is present or absent during an amplification reaction. In

certain embodiments, the labeled probes are designed to comprise a sequence that is the same as the sequence of the addressable portion or that is complementary to the sequence of the addressable portion.

[0183] In certain embodiments, one or more nucleic acid species are subjected to ligation and amplification reactions, either directly or via an intermediate, such as a cDNA target generated from an mRNA by reverse transcription. In certain embodiments, the initial nucleic acid comprises mRNA and a reverse transcription reaction may be performed to generate at least one cDNA, followed by at least one ligation reaction and at least one amplification reaction. In certain embodiments, DNA ligation probes hybridize to target RNA, and an RNA dependent DNA ligase is employed in a ligation reaction, followed by an amplification reaction. The ligation products and amplification products may be detected (or quantitated) using labeled probes.

[0184] In certain embodiments, for each target nucleic acid sequence to be detected, a ligation probe set, comprising at least one first probe and at least one second probe, is combined with the sample to form a ligation reaction composition. In certain embodiments, the ligation composition may further comprise a ligation agent. In certain embodiments, the first and second probes in each ligation probe set are suitable for ligation together and are designed to hybridize to adjacent sequences that are present in the target nucleic acid sequence. When the target nucleic acid sequence is present in the sample, the first and second probes will, under appropriate conditions, hybridize to adjacent regions on the target nucleic acid sequence (*see, e.g.,* probes 2 and 3 hybridized to target nucleic acid sequence 1 in Fig. 2A). In Figure 2A, the target nucleic acid sequence (1) is depicted as hybridized with a first probe (2), for illustration purposes shown here as

comprising a 5' primer-specific portion (25), an addressable portion (4), and a target-specific portion (15a), and a second probe (3) comprising a 3' primer-specific portion (35), a target-specific portion (15b) and a free 5' phosphate group ("P") for ligation.

[0185] In certain embodiments, the adjacently hybridized probes may, under appropriate conditions, be ligated together to form a ligation product (*see, e.g.,* ligation product 6 in Fig 2B). Figure 2B depicts the ligation product (6), generated from the ligation of the first probe (2) and the second probe (3). The ligation product (6) is shown comprising the 5' primer-specific portion (25), the addressable portion (4), and the 3' primer-specific portion (35). In certain embodiments, when the duplex comprising the target nucleic acid sequence (1) and the ligation product (6) is denatured, for example, by heating, the ligation product (6) is released.

[0186] In certain embodiments, one forms an amplification reaction composition comprising the ligation product 6, at least one primer set 7, a polymerase 8, and a labeled probe 26 (*see, e.g.,* Fig. 2C). The labeled probe 26 in the depicted embodiment is a 5'-nuclease fluorescent probe that comprises a quenching moiety (Q) linked to a fluorescent moiety (F) through an oligonucleotide link element that comprises a sequence complementary to the sequence of the addressable portion of the ligation product. In the first amplification cycle, the second primer 7', comprising a sequence complementary to the sequence of the 3' primer-specific portion 35 of the ligation product 6, hybridizes with the ligation product 6 and is extended, in the presence of DNA polymerase and deoxynucleoside triphosphates (dNTPs), in a template-dependent fashion. The 5'-nuclease activity of the polymerase results in cleavage of the 5'-nuclease fluorescent probe such that the fluorescent moiety (F) no longer is quenched by the

quenching moiety (Q) and a fluorescent signal is detected. Detection of the fluorescent signal from the 5'-nuclease fluorescent probe indicates the presence of the target nucleic acid sequence in the sample.

[0187] In certain embodiments, if no target nucleic acid sequence had been present in the sample, no ligation product comprising the addressable portion and the 5' and 3' primer-specific portions would have been formed during the ligation reaction. Accordingly, no labeled probe would bind to a ligation product or an amplification product and there would be no cleavage of a labeled probe during the amplification reaction. (Some of the labeled probes may hybridize to unligated ligation probes.) Thus, the absence of a detectable signal during or after the amplification reaction would indicate the absence of target nucleic acid sequence in the sample. In certain embodiments, ligation products may form even if the appropriate target nucleic acid sequence is not in the sample, but such ligation occurs to a measurably lesser extent than when the appropriate target nucleic acid sequence is in the sample. In certain such embodiments, one can set an appropriate threshold difference between detectable signal values to differentiate between samples that include the appropriate target nucleic acid sequence and samples that do not include the appropriate target nucleic acid sequence.

[0188] Certain embodiments may be substantially the same as those depicted in Figures 2A to 2C, except that the oligonucleotide link element of the 5'-nuclease fluorescent probe comprises the sequence of the addressable portion of the ligation product (rather than a sequence that is complementary to the sequence of the addressable portion). See, e.g., labeled probe 27 in Figures 2D and 2E.

[0189] In the first amplification cycle, the second primer 7', comprising a sequence complementary to the sequence of the 3' primer-specific portion 35 of the

ligation product 6, hybridizes with the ligation product 6 and is extended, in the presence of DNA polymerase and deoxynucleoside triphosphates (dNTPs), in a template-dependent fashion. The first amplification cycle generates a double-stranded product that comprises a complement of the 5' primer-specific portion (25) of the ligation product and a complement of the addressable portion (4) of the ligation product (see Figure 2D).

[0190] The double-stranded primer-extension product is denatured and subjected to one or more cycles of the polymerase chain reaction (PCR) including the labeled probe 27, which comprises an oligonucleotide link element that comprises the sequence of the addressable portion of the ligation product (see Figure 2E). A primer that comprises the sequence of the 5' primer-specific portion of the ligation product hybridizes with the amplification product that includes a sequence 25' that is complementary to the sequence of the 5' primer-specific portion and is extended, in the presence of DNA polymerase and deoxynucleoside triphosphates (dNTPs), in a template-dependent fashion. See, e.g., Figure 2E. The 5'-nuclease activity of the polymerase results in cleavage of the 5'-nuclease fluorescent probe such that the fluorescent moiety (F) no longer is quenched by the quenching moiety (Q) and a fluorescent signal is detected. Detection of the fluorescent signal from the 5'-nuclease fluorescent probe indicates the presence of the target nucleic acid sequence in the sample.

[0191] In certain embodiments, if no target nucleic acid sequence had been present in the sample, no ligation product comprising the addressable portion and the 5' and 3' primer-specific portions would have been formed during the ligation reaction. Thus, no amplification product comprising the complement of the addressable portion of such a ligation product would be formed. Accordingly, no

labeled probe would bind to a ligation product or an amplification product and there would be no cleavage of a labeled probe during the amplification reaction. Thus, the absence of a detectable signal during or after the amplification reaction would indicate the absence of target nucleic acid sequence in the sample. In certain embodiments, ligation products may form even if the appropriate target nucleic acid sequence is not in the sample, but such ligation occurs to a measurably lesser extent than when the appropriate target nucleic acid sequence is in the sample. In certain such embodiments, one can set an appropriate threshold difference between detectable signal values to differentiate between samples that include the appropriate target nucleic acid sequence and samples that do not include the appropriate target nucleic acid sequence.

[0192] When the amplification product exists as a double-stranded molecule in either of the embodiments in Figure 2, in certain embodiments, subsequent amplification cycles may exponentially amplify this molecule. In certain embodiments, one may quantitate the amount of target nucleic acid present in the sample by determining the level of intensity of the fluorescent signal.

[0193] As shown in Figure 3A, in certain embodiments, an mRNA is used to generate a cDNA copy 1'. The cDNA serves as a target nucleic acid sequence to which the first and second probes of the ligation probe set hybridize (see Figure 3B). The first probe 22 comprises a 5' primer-specific portion (25) and a target-specific portion 15a, and the second probe 23 comprises a target-specific portion 15b, an addressable portion 4, and a 3' primer-specific portion (35). Under appropriate conditions, the adjacently hybridized probes can form a ligation product 28 comprising a 5' primer-specific portion (25), the target-specific portions 15a and

15b, the addressable portion 4, and the 3' primer-specific portion (35) (see Figure 3C).

[0194] When the duplex formed by the target nucleic acid sequence 1' and the ligation product 28 is denatured, in certain embodiments by heating, the ligation product is released. In the presence of the appropriate primer set and under appropriate conditions, the 3' primer hybridizes with the 3' primer-specific portion 35 of the ligation product 28. The 3' primer is extended in the presence of DNA polymerase 8, generating a double-stranded product that comprises a complement (25') of the 5' primer-specific portion (25) of the ligation product and a complement (4') of the addressable portion (4) of the ligation product (see Figure 3D).

[0195] The double-stranded primer-extension product is denatured and subjected to one or more cycles of the polymerase chain reaction (PCR) including a labeled probe 27 (see, e.g., Figure 3E). The labeled probe 27 in the depicted embodiment is a 5'-nuclease fluorescent probe that comprises a quenching moiety (Q) linked to a fluorescent moiety (F) by an oligonucleotide that comprises the sequence of the addressable portion of the ligation product. A primer that comprises the sequence of the 5' primer-specific portion of the ligation product hybridizes with the amplification product that includes a sequence 25' that is complementary to the sequence of the 5' primer-specific portion and is extended, in the presence of DNA polymerase and deoxynucleoside triphosphates (dNTPs), in a template-dependent fashion. The 5'-nuclease activity of the polymerase results in cleavage of the 5'-nuclease fluorescent probe such that the fluorescent moiety (F) no longer is quenched by the quenching moiety (Q) and a fluorescent signal is detected (see, e.g., Figure 3E). Detection of the fluorescent signal from the 5'-

nuclease fluorescent probe indicates the presence of the target nucleic acid sequence in the sample.

[0196] In certain embodiments, if no target nucleic acid sequence had been present in the sample, no ligation product comprising the addressable portion and the 5' and 3' primer-specific portions would have been formed during the ligation reaction. Thus, no amplification product comprising the complement of such a ligation product would be formed. Accordingly, no labeled probe would bind to a ligation product or an amplification product and there would be no cleavage of a labeled probe during the amplification reaction. Thus, the absence of a detectable signal during or after the amplification reaction would indicate the absence of target nucleic acid sequence in the sample. In certain embodiments, ligation products may form even if the appropriate target nucleic acid sequence is not in the sample, but such ligation occurs to a measurably lesser extent than when the appropriate target nucleic acid sequence is in the sample. In certain such embodiments, one can set an appropriate threshold difference between detectable signal values to differentiate between samples that include the appropriate target nucleic acid sequence and samples that do not include the appropriate target nucleic acid sequence.

[0197] Certain embodiments may be substantially the same as those depicted in Figures 3A to 3E, except that the oligonucleotide link element of the 5'-nuclease fluorescent probe comprises a sequence that is complementary to the sequence of the addressable portion of the ligation product (rather than the sequence of the addressable portion). See, e.g., labeled probe 26 in Figure 3F.

[0198] In the first amplification cycle, the second primer 7', comprising a sequence complementary to the sequence of the 3' primer-specific portion 35 of the

ligation product 28, hybridizes with the ligation product 28 and is extended, in the presence of DNA polymerase and deoxynucleoside triphosphates (dNTPs), in a template-dependent fashion. The 5'-nuclease activity of the polymerase results in cleavage of the 5'-nuclease fluorescent probe such that the fluorescent moiety (F) no longer is quenched by the quenching moiety (Q) and a fluorescent signal is detected.

[0199] In certain embodiments, if unligated second ligation probes have been substantially removed from the composition after the ligation reaction, detection of the fluorescent signal from the first amplification cycle indicates the presence of the target nucleic acid sequence in the sample. In certain embodiments, after the ligation reaction, one may substantially remove unligated second probes by exposing the composition to nucleic acids on a solid phase that are complementary to a sequence that is included on the first ligation probe, but that is not included on the second ligation probe. One may then separate the hybridized ligation products and unligated first ligation probes on the solid phase from the unligated second ligation probes.

[0200] If the unligated second ligation probes have not been substantially removed from the composition after the ligation reaction, detection of the fluorescent signal from the first amplification cycle does not necessarily indicate the presence of target nucleic acid in the sample. In such embodiments, labeled probes will hybridize to both unligated second ligation probes and ligation products. Also, the 5'-nuclease activity of the polymerase results in cleavage of the 5'-nuclease fluorescent probes that are hybridized to both the unligated second ligation probes and ligation products. Thus, the same signal would be detected whether or not any ligation product is present.

[0201] Subsequent cycles of amplification, however, may be employed in such embodiments to detect the presence or absence of (or to quantitate) target nucleic acid sequence. If no ligation product is present, the quantity of sequences that comprise an addressable sequence will not increase with subsequent cycles of amplification. Only the initial quantity of unligated second ligation probes will interact with the labeled probes to emit a signal.

[0202] In contrast, subsequent amplification cycles involving a composition that includes ligation products will result in an increased quantity of sequences that comprise the addressable portion. Thus, the quantity of amplification product with which the labeled probes interact increases. Thus, in certain embodiments, one can set the threshold difference between detectable signal values to differentiate between samples that include ligation product and samples that do not include ligation product. In certain embodiments, ligation products may form even if the appropriate target nucleic acid sequence is not in the sample, but such ligation occurs to a measurably lesser extent than when the appropriate target nucleic acid sequence is in the sample. In certain such embodiments, one can set an appropriate threshold difference between detectable signal values to differentiate between samples that include the appropriate target nucleic acid sequence and samples that do not include the appropriate target nucleic acid sequence.

[0203] The embodiments depicted in Figure 3 may be modified by simply using target DNA in a sample rather than using cDNA resulting from reverse transcription of RNA. Also, the embodiments depicted in Figure 3 may be modified by using the RNA as the target nucleic acid sequence to which the ligation probes hybridize.

[0204] In this application, whenever one employs an amplification reaction to determine whether there is a threshold difference in signal value from a labeled probe, the amplification reaction is carried out in a manner that will result in such a threshold difference if the target sequence that is being sought is included in the sample. The following nonlimiting exemplary embodiments illustrate this concept.

[0205] In a first exemplary embodiment, one employs a ligation probe set that comprises: a first probe that comprises a 5' primer specific portion and a target-specific portion; and a second probe that comprises a target specific portion, an addressable portion, and a 3' primer-specific portion. If the target nucleic acid is present in the sample, the first and second probes are ligated together to form a ligation product during a ligation reaction. The ligation product comprises the 5' primer-specific portion, the two target-specific portions, the addressable portion, and the 3' primer-specific portion.

[0206] In this embodiment, one forms an amplification reaction composition comprising the ligation product, a 5' nuclease fluorescent probe that comprises the sequence of the addressable portion, and a set of appropriate primers for the 5' and 3' primer-specific portions. The 5' nuclease fluorescent probe has a first detectable signal value when it is not hybridized to a complementary sequence. If one employs PCR as the amplification reaction, the first cycle of amplification will not result in a threshold difference between the first detectable signal value and a second detectable signal value during and/or after the first cycle of amplification. No threshold difference is detected, since the 5' nuclease fluorescent probe has the same sequence as the addressable portion of the ligation product and thus will not hybridize to the addressable portion. Thus, there will be no cleavage of the 5' nuclease fluorescent probe during the first cycle of amplification.

[0207] The first cycle of amplification, however, results in an amplification product that comprises the complement of the addressable portion and the complement of the 5' primer-specific portion at its 3' end. Thus, the 5' nuclease fluorescent probe will hybridize to the amplification product and will be cleaved during the second cycle of amplification. Thus, in this exemplary embodiment, the second cycle of amplification results in a threshold difference between the first detectable signal value and the second detectable signal value during and/or after the second cycle of amplification. Thus, in such embodiments, the amplification reaction used to determine whether there is a threshold difference in signal value comprises at least two cycles of PCR amplification.

[0208] In a second exemplary embodiment, one employs a ligation probe set that comprises: a first probe that comprises a 5' primer specific portion, an addressable portion, and a target-specific portion; and a second probe that comprises a target specific portion and a 3' primer-specific portion. If the target nucleic acid is present in the sample, the first and second probes are ligated together to form a ligation product during a ligation reaction. The ligation product comprises the 5' primer-specific portion, the addressable portion, the two target-specific portions, and the 3' primer-specific portion.

[0209] In this embodiment, one forms an amplification reaction composition comprising the ligation product, a 5' nuclease fluorescent probe that comprises a sequence complementary to sequence of the addressable portion, and a set of appropriate primers for the 5' and 3' primer-specific portions. The 5' nuclease fluorescent probe has a first detectable signal value when it is not hybridized to a complementary sequence. If one employs PCR as the amplification reaction, the first cycle of amplification will result in a threshold difference between the first

detectable signal value and the second detectable signal value during and/or after the first cycle of amplification. A threshold difference is detected since the 5' nuclease fluorescent probe has a sequence complementary to sequence of the addressable portion of the ligation product and thus hybridizes to the addressable portion, and the first cycle of amplification results in cleavage of the 5' nuclease fluorescent probe. Thus, in such embodiments, the amplification reaction used to determine whether there is a threshold difference in signal value comprises at least one cycle of PCR amplification.

[0210] In a third exemplary embodiment, one employs a ligation probe set that comprises: a first probe that comprises a 5' primer specific portion and a target-specific portion; and a second probe that comprises a target specific portion, an addressable portion, and a 3' primer-specific portion. If the target nucleic acid is present in the sample, the first and second probes are ligated together to form a ligation product during a ligation reaction. The ligation product comprises the 5' primer-specific portion, the two target-specific portions, the addressable portion, and the 3' primer-specific portion.

[0211] In this embodiment, one forms an amplification reaction composition comprising the ligation product, a hybridization dependent probe that comprises the sequence of the addressable portion, and a set of appropriate primers for the 5' and 3' primer-specific portions. The hybridization dependent probe has a first detectable signal value when it is not hybridized to a complementary sequence. In this embodiment, PCR is used as the amplification reaction.

[0212] If unligated probes are not substantially removed from the amplification reaction composition prior to the first cycle of amplification, no threshold difference is detected during and/or after the first cycle. No threshold

difference is detected, since, whether or not the sought ligation product is present, the first cycle of amplification will result in the same number of amplification products to which the hybridization dependent probes will hybridize. Both the unligated probes and the ligation products in such embodiments will comprise the same 3' primer-specific portion that will initiate extension in the first cycle of amplification and will comprise the same addressable portion. Thus, after the first cycle of amplification, when the hybridization dependent probes hybridize to the complement of the addressable portion on the amplification products, the same signal value will result whether or not the ligation product is present.

[0213] A threshold difference in detectable signal value, however, will result in subsequent cycles of amplification when amplification products with sequences complementary to the sequence of the addressable portion increase exponentially when the ligation product is amplified. In such subsequent cycles, if no ligation product is present, such amplification products will only increase linearly from the presence of the unligated probes. Such linear amplification occurs, since, unlike the ligation product, the unligated probes do not comprise 5' primer-specific portions.

[0214] In a fourth exemplary embodiment, one employs a ligation probe set that comprises: a first probe that comprises a 5' primer specific portion and a target-specific portion; and a second probe that comprises a target specific portion, an addressable portion, and a 3' primer-specific portion. If the target nucleic acid is present in the sample, the first and second probes are ligated together to form a ligation product during a ligation reaction. The ligation product comprises the 5' primer-specific portion, the two target-specific portions, the addressable portion, and the 3' primer-specific portion.

[0215] In this embodiment, one forms an amplification reaction composition comprising the ligation product, a hybridization dependent probe that comprises a sequence that is complementary to the sequence of the addressable portion, and a set of appropriate primers for the 5' and 3' primer-specific portions. Also, in this embodiment, a substantial portion of the hybridization dependent probes are not cleaved during a cycle of an amplification reaction. A "substantial portion of the hybridization dependent probes are not cleaved" refers to a portion of the total number of hybridization dependent probes that are designed to hybridize to a given nucleic sequence that is being amplified, and it does not refer to a portion of an individual probe. In certain embodiments, "a substantial portion of hybridization dependent probes that are not cleaved" means that at least 90% of the hybridization dependent probes are not cleaved. In certain embodiments, at least 95% of the hybridization dependent probes are not cleaved. The hybridization dependent probe has a first detectable signal value when it is not hybridized to a complementary sequence. In this embodiment, PCR is used as the amplification reaction.

[0216] If unligated probes are not substantially removed from the amplification reaction composition prior to the first cycle of amplification, no threshold difference is detected during and/or after the first cycle. No threshold difference is detected, since the hybridization dependent probes will hybridize to both unligated second probes and ligation products. The first cycle of amplification results in amplification products that have sequences that are complementary to the sequence of the addressable portion of the ligation product. Thus, the hybridization dependent probes do not hybridize to any amplification products produced in the first cycle of amplification.

[0217] A threshold difference in detectable signal value, however, will result after the second cycle of amplification, since the second cycle results in an increase of DNA that comprises the sequence of the addressable portion only if ligation product is present. Thus, in such embodiments, the amplification reaction used to determine whether there is a threshold difference in signal value comprises at least two cycles of PCR amplification.

[0218] According to certain embodiments, the first and second probes in each ligation probe set are designed to be complementary to the sequences immediately flanking the pivotal nucleotide of the target sequence (see, e.g., probes A, B, and Z in Fig. 6(1)). In the embodiment shown in Fig. 6, two first probes A and B of a ligation probe set will comprise a different nucleotide at the pivotal complement and a different addressable portion for each different nucleotide at the pivotal complement. One forms a ligation reaction composition comprising the probe set and the sample.

[0219] When the target sequence is present in the sample, the first and second probes will hybridize, under appropriate conditions, to adjacent regions on the target (see, e.g., Fig. 6(2)). When the pivotal complement is base-paired to the target, in the presence of an appropriate ligation agent, two adjacently hybridized probes may be ligated together to form a ligation product (see, e.g., Fig 6(3)). In certain embodiments, if the pivotal complement of a first probe is not base-paired to the target, no ligation product comprising that mismatched probe will be formed (see, e.g., probe B in Figs. 6(2) to 6(4).

[0220] In Figs. 6(2) and 6(3), the first probe B is not hybridized to a target. In certain embodiments, the failure of a probe with a mismatched terminal pivotal complement to ligate to a second probe may arise from the failure of the probe with

the mismatch to hybridize to the target under the conditions employed. In certain embodiments, the failure of a probe with a mismatched terminal pivotal complement to ligate to a second probe may arise when that probe with the mismatch is hybridized to the target, but the nucleotide at the pivotal complement is not base-paired to the target.

[0221] In certain embodiments, the reaction volume that is subjected to the ligation reaction forms a test composition. In certain embodiments, one then forms an amplification reaction composition comprising the test composition, at least one primer set, a polymerase, and a different labeled probe (LBP-A and LBP-B) for each different first probe, wherein the different labeled probes can provide detectably different signals (see, e.g., Fig. 6(4)). The labeled probes in the depicted embodiment are different 5'-nuclease fluorescent probes that comprise a quenching moiety (Q) linked to a detectably different fluorescent moiety (F) through a different oligonucleotide link element. The different oligonucleotide link elements comprise a sequence that is complementary to one of the different addressable portions of the different first ligation probes.

[0222] In the depicted embodiment, the first labeled probe (LBP-A) comprises a first fluorescent moiety (FA) that is linked to the quenching moiety (Q) through an oligonucleotide link element that comprises a sequence complementary to the sequence of the addressable portion (ASP-A) of the first probe A. In the depicted embodiment, the second labeled probe (LBP-B) comprises a second fluorescent moiety (FB) that is linked to the quenching moiety (Q) through an oligonucleotide link element that comprises a sequence that is complementary to the sequence of the addressable portion (ASP-B) of the first probe B. The

fluorescent moieties of each of the different labeled probes emit detectably different signals from one another when they are not quenched by the quenching moiety.

[0223] In certain appropriate salts, buffers, and nucleotide triphosphates, the amplification reaction composition is subjected to at least one cycle of amplification. In the first amplification cycle, the second primer (P2), which comprises a sequence complementary to the sequence of the 3' primer-specific portion of the ligation product, hybridizes with the ligation product and is extended in a template-dependent fashion to create a double-stranded molecule.

[0224] Also during the first amplification cycle, the 5'-nuclease activity of the polymerase results in cleavage of the labeled probe that is hybridized to the addressable portion of the ligation product (see, e.g., labeled probe LBP-A in Fig. 6(5). Cleavage results in the fluorescent moiety (FA) no longer being quenched by the quenching moiety (Q) and a fluorescent signal is detected. Detection of the fluorescent signal from fluorescent moiety (FA) indicates the presence of the target nucleic acid sequence in the sample that has a pivotal nucleotide (A) that is complementary to the nucleotide (T) at the pivotal complement of the ligation product.

[0225] In this example, no target nucleic acid sequence in the sample has a pivotal nucleotide (C) that is complementary to the nucleotide of the pivotal complement of probe B. Thus, in this example, no ligation product comprising the addressable portion of probe B and the 3' primer-specific portion is formed. Accordingly, no labeled probe (LBP-B) comprising fluorescent moiety (FB) would bind to a ligation product or an amplification product and there would be no cleavage of a labeled probe (LBP-B) during the amplification reaction. Thus, the absence of a detectable signal from fluorescent moiety (FB) during or after the

amplification reaction would indicate the absence of target nucleic acid sequence having pivotal nucleotide (C) in the sample. In certain embodiments, ligation of probes with a pivotal complement that is not complementary to the pivotal nucleotide may occur, but such ligation occurs to a measurably lesser extent than ligation of probes with a pivotal complement that is complementary to the pivotal nucleotide. In certain such embodiments, one can set an appropriate threshold difference between detectable signal values to differentiate between samples that include the appropriate target nucleic acid sequence and samples that do not include the appropriate target nucleic acid sequence.

[0226] In certain embodiments, when a 5'-nuclease probe hybridizes to an addressable portion or the complement of an addressable portion, the quenching moiety may be separated enough from the signal moiety such that a signal may be detected. In certain embodiments, the detectable signal value of such a signal (prior to cleavage) is less than the detectable signal value after cleavage of the 5'-nuclease probe. Thus, in certain such embodiments, one may set a threshold difference in detectable signal values such that a signal value that is detected after hybridization of the 5'-nuclease probe to a given sequence without cleavage does not result in the set threshold difference. A signal value that is detected with cleavage of the 5'-nuclease probe, however, will result in the set threshold difference.

[0227] In certain embodiments, subsequent amplification cycles may result in exponential amplification (see, e.g., Fig. 6(4)-(11)). Thus, with each cycle the signal value that is detected from the cleavage of labeled probes (LBP-A) will increase, while the signal value that is detected from labeled probes (LBP-B) will stay substantially the same.

[0228] In certain embodiments, a single-stranded amplification product is synthesized by, for example, without limitation, asymmetric PCR, asynchronous PCR, primer extension, or asymmetric reamplification. In exemplary embodiments of asymmetric PCR, the amplification reaction composition is prepared with at least one primer set, wherein either the at least one first primer, or the at least one second primer, but not both, are added in excess. Thus, in certain embodiments, the excess primer to limiting primer ratio may be approximately 100:1, respectively. One of ordinary skill in the art will recognize that the optimal amounts of the primers according to certain embodiments may be determined empirically. In certain embodiments, amounts will range from about 2 to 50 nM for the limiting primer, and from about 100 to 900 nM for the primer in excess. Empirically, in certain embodiments, the concentration of one primer in the primer set is typically kept below 5 pmol per 100 μl of amplification reaction composition.

[0229] Since both primers are initially present in substantial excess at the beginning of the PCR reaction in certain embodiments, both strands are exponentially amplified. In certain embodiments, prior to completing all of the cycles of amplification, however, the limiting primer is exhausted. During the subsequent cycles of amplification, only one strand is amplified, thus generating an excess of single-stranded amplification products.

[0230] For example, but without limitation, in certain embodiments, after approximately 40 to 45 cycles of amplification are performed, the amplification process is completed with a long extension step. In certain embodiments, the limiting primer is typically exhausted by the 25th cycle of amplification. During subsequent cycles of amplification only one strand of the amplification product is produced due to the presence of only one primer of the primer set. In certain

embodiments, the labeled probe is a 5' nuclease probe that is designed to hybridize with a template strand that is not being produced during such subsequent cycles, such that each subsequent cycle results in an additional amount of signal. In certain embodiments, the labeled probe is a hybridization dependent probe that is designed to hybridize with a template strand that is being produced during such subsequent cycles, such that each subsequent cycle results in an additional amount of signal.

[0231] In certain exemplary asymmetric reamplification protocols, an air-dried first amplification composition containing double-stranded amplification product, is resuspended in 30 μl of 0.1 x TE buffer, pH 8.0. The second amplification reaction composition is prepared by combining two microliters of the resuspended amplification product in a 0.2 ml MicroAmp reaction tube with 9 μl sterile filtered deionized water, 18 μl AmpliTaq Gold® mix (PE Biosystems, Foster City, CA), an appropriate amount of labeled probe, and 20-40 pmol of either the at least one first primer or the at least one second primer suspended in 1 μl 1xTE buffer.

[0232] The tubes are heated to 95° C for 12 minutes, then cycled for ten cycles of (94°C for 15 seconds, 60°C for 15 seconds, and 72° C for 30 seconds), followed by twenty-five cycles of (89°C for 15 seconds, 53° C for 15 seconds, and 72° C for 30 seconds), and then 45 minutes at 60° C. The labeled probes are designed such that the detectable signal changes during the subsequent reamplification procedure, if the corresponding ligation product is present prior to the initial amplification reaction.

[0233] For example, in certain embodiments, one will form a ligation product from: a first probe that comprises a 5' primer-specific portion, an addressable

portion, and a target-specific portion; and a second probe comprising a target specific portion and a 3' primer-specific portion. The primer set will include a first primer that comprises the sequence of the 5' primer-specific portion and a second primer that comprises a sequence that is complementary to the sequence of the 3' primer-specific portion. The labeled probe will comprise a sequence that is complementary to the sequence of the addressable portion and the second primer will be included in excess of the first primer.

[0234] In certain embodiments, a double-stranded amplification product is generated and subsequently converted into single-stranded sequences. Processes for converting double-stranded nucleic acid into single-stranded sequences include, without limitation, heat denaturation, chemical denaturation, and exonuclease digestion. Detailed protocols for synthesizing single-stranded nucleic acid molecules or converting double-stranded nucleic acid into single-stranded sequences can be found, among other places, in Ausbel et al., Sambrook et al., the Novagen Strandase™ product insert (Novagen, Madison, WI), and Sambrook and Russell.

[0235] In certain embodiments, the methods of the invention comprise universal primers, universal primer sets, or both. In certain embodiments, one may use a single universal primer set for any number of amplification reactions for different target sequences.

[0236] In certain embodiments, one may employ the same two different addressable portions for the two different allelic options at more than one locus. In certain such embodiments, one may distinguish between the different loci by employing a different reaction composition for each locus.

[0237] Thus, if one wants to determine a single nucleotide difference in the alleles at three different biallelic loci, in certain such embodiments, one may employ three different reaction compositions that each have a different ligation probe set specific for the two options at each locus. Figures 7A-7C illustrates certain such embodiments in which one employs three different reaction compositions for three biallelic loci. In Figures 7A-7C, there is a different probe set for each of the three different loci. Each probe set comprises two first probes for the two different alleles at each locus. Each of the first probes of each probe set comprises the same 5' primer-specific portion (P-SP(A)), a target-specific portion that is complementary to a portion of the given locus and includes a different nucleotide at the pivotal complement (A or G for the first locus; T or G for the second locus; G or C for the third locus), and a different addressable portion (AP1 or AP2) corresponding to one of the two allelic nucleotide options for each locus. The same set of addressable portions (AP1 and AP2) can be used on the two first probes of each of the three different probe sets. Each of the second probes of each probe set comprises the same 3' primer-specific portion (P-SP(Z)) and a different target-specific portion for each different locus.

[0238] In certain embodiments shown in Figures 7A-7C, after separate ligation reactions for each locus, one can perform three separate amplification reactions for each locus with the same primer set (PA) and (PZ) and the same two labeled probes (LBP-1, which comprises a sequence that is complementary to (or is the same as) the sequence of the addressable portion AP1; and LBP-2, which comprises a sequence that is complementary to (or is the same as) the sequence of the addressable portion AP2). Also, the two different labeled probes provide two detectably different signals.

[0239] Thus, in this example, if amplification results in a threshold difference in detectable signal value from both labeled probes (LBP-1 and LBP-2) in all three reaction compositions, one would conclude that the sample was heterozygous at all three loci. Another possible result from the amplification reactions may be as follows: the first amplification reaction composition results in a threshold difference in detectable signal value from labeled probe LBP-1, the second amplification reaction composition results in a threshold difference in detectable signal value from labeled probes LBP-1 and LBP-2, and the third amplification reaction composition results in a threshold difference in detectable signal value from labeled probe LBP-1. One would conclude from such results that the sample is homozygous at locus 1 with (C) as the pivotal nucleotide, heterozygous at locus 2, and homozygous at locus 3 with (G) as the pivotal nucleotide.

[0240] In certain embodiments, one may analyze many different target sequences employing specific different probe sets in separate reaction compositions. For example, one could employ a 96 well plate with 96 different ligation probe sets for 96 different target nucleic acid sequences. In certain embodiments, one may want to detect the presence or absence of (or to quantitate) a single target nucleic acid sequence with each of the 96 probe sets. In certain such embodiments, one may employ the same set of two primers and the same labeled probe in each of the different 96 wells to obtain results for 96 different target sequences.

[0241] In certain embodiments, one may want to detect the presence or absence of (or to quantitate) two different alleles at 96 different loci with 96 different ligation probe sets. In certain embodiments, each probe set comprises two first probes and one second probe. In certain embodiments, each of the first probes of

each probe set comprises a target-specific portion that is complementary to a portion of the given locus and includes a different nucleotide at the pivotal complement, and one of two different addressable portions corresponding to one of the two allelic nucleotide options for each locus. In certain embodiments, the same two different addressable portions can be used on the two first probes of each of the 96 probe sets. In certain embodiments, each of the second probes of each probe set comprises a different target-specific portion for each locus. In certain embodiments, the two first probes of each of the 96 probe sets may further comprise the same primer-specific portion. In certain embodiments, each of the second probes of each of the 96 probe sets may further comprise another primer-specific portion.

[0242] In certain such embodiments, after ligation, one may perform 96 separate amplification reactions in the 96 different wells. In certain such embodiments, one may use in all of the 96 wells the same primer set and the same two labeled probes. One labeled probe may comprise a sequence that is complementary to (or is the same as) one of the sequences of the two addressable portions, and the other labeled probe may comprise a sequence that is complementary to (or is the same as) the sequence of the other of the two addressable portions. Also, the two different labeled probes provide two detectably different signals. One may detect which allele or alleles are present in each of 96 wells by detecting a change in detectable signal value from the labeled probes.

[0243] The skilled artisan will understand that, in various embodiments, ligation probes can be designed with a pivotal complement at any location in either the first probe or the second probe. Additionally, in certain embodiments, ligation probes may comprise multiple pivotal complements.

[0244] In certain embodiments that employ ligation probe sets that comprise multiple first probes for a given locus that comprise target-specific portions with different pivotal complements, the target-specific portions of each of the different first probes for a given locus may have the same sequence except for a different nucleotide at the pivotal complement. In certain embodiments, the target-specific portions of each of the first probes for a given locus may have a different nucleotide at the pivotal complement and may have different length sequences 5' to the pivotal complement. In certain such embodiments, such target-specific portion sequences 5' to the pivotal complement may all be complementary to a portion of the same locus nucleic acid sequence adjacent to the pivotal nucleotide, but may have different lengths. For example, in such embodiments in which there are two different first probes, the target-specific portion sequences 5' to the pivotal complement may be the same except one of them may have one or more additional nucleotides at the 5' end of the target-specific portion.

[0245] In certain embodiments that employ ligation probe sets that comprise multiple second probes for a given locus that comprise target-specific portions with different pivotal complements, the target-specific portions of each of the different second probes for a given locus may have the same sequence except for a different nucleotide at the pivotal complement. In certain embodiments, the target-specific portions of each of the second probes for a given locus may have a different nucleotide at the pivotal complement and may have different length sequences 3' to the pivotal complement. In certain such embodiments, such target-specific portion sequences 3' to the pivotal complement may all be complementary to a portion of the same locus nucleic acid sequence adjacent to the pivotal nucleotide, but may have different lengths. For example, in such embodiments in

which there are two different second probes, the target-specific portion sequences 3' to the pivotal complement may be the same except one of them may have one or more additional nucleotides at the 3' end of the target-specific portion.

[0246] In certain embodiments, the number of ligation probes used to detect any number of target sequences, is the product of the number of targets to be detected times the number of alleles to be detected per target plus one (i.e., (number of target sequences x [number of alleles + 1]). Thus, to detect 3 biallelic sequences, for example, nine probes are used (3 x [2 + 1]). In certain embodiments, to detect 4 triallelic sequences, 16 probes are used (4 x [3 + 1]), and so forth.

[0247] The significance of the decrease in the number of primers and labeled probes in certain embodiments, and therefore the cost and number of manipulations, becomes readily apparent when performing genetic screening of an individual for a large number of multiallelic loci or of many individuals. In certain embodiments, to amplify the ligation product of a target sequence, two primers are used. One primer is complementary to the sequence of the 3' primer-specific portion of the ligation products, and one primer comprises the sequence of the 5' primer-specific portion. Using certain conventional methods, one employs three different primers for each different ligation product. Thus, to amplify the ligation products for three biallelic loci potentially present in an individual using certain conventional methodology, one would use 9 (3n, where n=3) primers.

[0248] In contrast, certain embodiments of the present invention can effectively reduce this number to as few as two amplification primers. According to certain embodiments of the present invention, as few as two "universal" primers, can be used to amplify one or more ligation or amplification products, since the

probes may be designed to share primer-specific portions but comprise different addressable portions. A sample containing 100 possible biallelic loci would require 200 primers in certain conventional detection methods, yet only 2 universal primers can be used in certain embodiments of the present invention.

[0249] Also, if one were to use certain conventional methods employing labeled probes, a different labeled probe for each different allele at each different locus would be used. According to certain embodiments of the present invention, one can employ two labeled probes to detect the sequence of one or more different loci. For example, in certain conventional methods, one would use 200 different labeled probes to detect the 200 possible sequences at 100 biallelic loci. Using certain embodiments of the present invention, one can use 2 labeled probes to detect 200 possible sequences at 100 biallelic loci.

E. Certain Exemplary Applications

[0250] In certain embodiments, when the gene expression levels for several target nucleic acid sequences for a sample are known, a gene expression profile for that sample can be compiled and compared with other samples. For example, but without limitation, samples may be obtained from two aliquots of cells from the same cell population, wherein one aliquot was grown in the presence of a chemical compound or drug and the other aliquot was not. By comparing the gene expression profiles for cells grown in the presence of drug with those grown in the absence of drug, one may be able to determine the drug effect on the expression of particular target genes.

[0251] In certain embodiments, one may quantitate the amount of mRNA encoding a particular protein within a cell to determine a particular condition of an

individual. For example, the protein insulin, among other things, regulates the level of blood glucose. The amount of insulin that is produced in an individual can determine whether that individual is healthy or not. Insulin deficiency results in diabetes, a potentially fatal disease. Diabetic individuals typically have low levels of insulin mRNA and thus will produce low levels of insulin, while healthy individuals typically have higher levels of insulin mRNA and produce normal levels of insulin.

[0252] Another human disease typically due to abnormally low gene expression is Tay-Sachs disease. Children with Tay-Sachs disease lack, or are deficient in, a protein(s) required for sphingolipid breakdown. These children, therefore, have abnormally high levels of sphingolipids causing nervous system disorders that may result in death.

[0253] In certain embodiments, it is useful to identify and detect additional genetic-based diseases/disorders that are caused by gene over- or under-expression. Additionally, cancer and certain other known diseases or disorders may be detected by, or are related to, the over- or under-expression of certain genes. For example, men with prostate cancer typically produce abnormally high levels of prostate specific antigen (PSA); and proteins from tumor suppressor genes are believed to play critical roles in the development of many types of cancer.

[0254] Using nucleic acid technology, in certain embodiments, minute amounts of a biological sample can typically provide sufficient material to simultaneously test for many different diseases, disorders, and predispositions. Additionally, there are numerous other situations where it would be desirable to quantify the amount of specific target nucleic acids, in certain instances mRNA, in a cell or organism, a process sometimes referred to as "gene expression profiling."

When the quantity of a particular target nucleic acid within, for example, a specific cell-type or tissue, or an individual is known, in certain cases one may start to compile a gene expression profile for that cell-type, tissue, or individual. Comparing an individual's gene expression profile with known expression profiles may allow the diagnosis of certain diseases or disorders in certain cases. Predispositions or the susceptibility to developing certain diseases or disorders in the future may also be identified by evaluating gene expression profiles in certain cases. Gene expression profile analysis may also be useful for, among other things, genetic counseling and forensic testing in certain cases.

F. Certain Exemplary Kits

[0255] In certain embodiments, the invention also provides kits designed to expedite performing certain methods. In certain embodiments, kits serve to expedite the performance of the methods of interest by assembling two or more components used in carrying out the methods. In certain embodiments, kits may contain components in pre-measured unit amounts to minimize the need for measurements by end-users. In certain embodiments, kits may include instructions for performing one or more methods of the invention. In certain embodiments, the kit components are optimized to operate in conjunction with one another.

[0256] In certain embodiments, a kit for detecting at least one target nucleic acid sequence in a sample is provided. In certain embodiments, a kit comprises: a ligation probe set for each target sequence, the probe set comprising (a) at least one first probe, comprising a target-specific portion and a 5' primer-specific portion, wherein the 5' primer-specific portion comprises a sequence, and (b) at least one second probe, comprising a target-specific portion and a 3' primer-specific portion,

wherein the 3' primer-specific portion comprises a sequence. The probes in each set are suitable for ligation together when hybridized adjacent to one another on a complementary target sequence. One probe in each probe set further comprises an addressable portion located between the primer-specific portion and the target-specific portion, wherein the addressable portion comprises a sequence. In certain embodiments, the kit further comprises a labeled probe comprising the sequence of the addressable portion or comprising a sequence complementary to the sequence of the addressable portion.

[0257] In certain embodiments, the kit comprises a labeled probe that has a first detectable signal value when it is not hybridized to a complementary sequence and a second detectable signal value of the labeled probe can be detected at least one of during and after an amplification reaction. In certain embodiments, a threshold difference between the first detectable signal value and the second detectable signal value indicates the presence of the target nucleic acid sequence, and no threshold difference between the first detectable signal value and the second detectable signal value indicates the absence of the target nucleic acid sequence.

[0258] In certain embodiments, kits further comprise primers. In certain embodiments, kits further comprise at least one primer set comprising (i) at least one first primer comprising the sequence of the 5' primer-specific portion of the at least one first probe, and (ii) at least one second primer comprising a sequence complementary to the sequence of the 3' primer-specific portion of the at least one second probe.

[0259] In certain embodiments, kits comprise one or more additional components, including, without limitation, at least one of: at least one polymerase,

at least one transcriptase, at least one ligation agent, oligonucleotide triphosphates, nucleotide analogs, reaction buffers, salts, ions, and stabilizers. In certain embodiments, kits comprise one or more reagents for purifying the ligation products, including, without limitation, at least one of dialysis membranes, chromatographic compounds, supports, and oligonucleotides.

[0260] The following examples are intended for illustration purposes only, and should not be construed as limiting the scope of the invention in any way.

Example 1

[0261] The following Table 1 is referred to throughout the following Example 1:

TABLE 1

Probe Set For Assay 1

First Probe-CYC (1) 5'**TTGCCTGCTCGACTTAGA**TCAAAGGAGACGCGG*CTGCTTTCAGCCTCAT*3' (SEQ ID NO:1)

First Probe-RNA(1) 5'**TTGCCTGCTCGACTTAGA**GGGTCACAGTAGGTGG*TGCTTTCAGCCTCAC*3' (SEQ ID NO:2)

Second Probe (1) 5'P-*GGGGATAGTGGCTGC***ATCACTGGATAGCGACGT**3' (SEQ ID NO:3)

Probe Set For Assay 2

First Probe-CYC (2) 5'**TTGCCTGCTCGACTTAGA**TCAAAGGAGACGCGG*CAGTGGTTTTCCAACG*3' (SEQ ID NO:4)

First Probe-RNA (2) 5'**TTGCCTGCTCGACTTAGA**GGGTCACAGTAGGTGG*ACAGTGGTTTTCCAACA*3' (SEQ ID NO:5)

Second Probe (2) 5'P-*TGAACACACCGGGT***ATCACTGGATAGCGACGT**3' (SEQ ID NO:6)

PCR Primers

Forward Primer 5'TTGCCTGCTCGACTTAGA3' (SEQ ID NO:7)

Reverse Primer 5'ACGTCGCTATCCAGTGAT3' (SEQ ID NO:8)

TaqMan® Probe Sequences

CYCLOPHILIN: 5' CCGCGTCTCCTTTGA3'-MGBNFQ (labeled with VIC) (SEQ ID NO:9)

RNASE P: 5' CCACCTACTGTGACCC-MGBNFQ (labeled with FAM) (SEQ ID NO:10)

(MGB = minor groove binder and NFQ = nonfluorescent quencher, which are both included on TaqMan® probes available from Applied Biosystems, Foster City, CA)

A. Ligation probes

[0262] In these examples, a ligation probe set for each target nucleic acid sequence comprised first and second ligation probes designed to adjacently hybridize to the appropriate target nucleic acid sequence. These adjacently hybridized probes were, under appropriate conditions, ligated to form a ligation product.

[0263] This illustrative embodiment used two different ligation probe sets for detecting two biallelic loci. Three different samples of genomic DNA were tested. Table 1 shows the two probe sets that were used. Table 1 also shows the two Taqman® probes that were used in these examples. The ligation probes included a target-specific portion, shown in italic letters in Table 1. As shown by bold letters in Table 1, the ligation probes also included universal primer-specific portion sequences (18 nucleotides at the 5' end of the first listed probes in each probe set and 18 nucleotides at the 3' end of the second listed probe in each probe set). As shown by underlined letters in Table 1, the first two probes in each ligation probe set also included the same two different addressable portions that are complementary to the different sequences of the two TaqMan® probes.

[0264] The ligation probes were synthesized using conventional automated DNA synthesis chemistry.

B. Exemplary Ligation Reactions (Oligonucl otid Ligation Assay "OLA")

[0265] Ligation reactions were performed in separate reaction volumes with each of the two different ligation probe sets shown in Table 1. The concentrations of the component materials prior to forming the ligation reaction composition are shown below in Table 2.

TABLE 2

Component Materials	Concentration
Thermus aquaticus (*Taq*) DNA Ligase	40 units / μL
10X OLA Buffer 2 Mixture: pH 7.5 @ 50° C	
- Sodium(3-[N-Morpholino]propanesulfonate) (MOPS)	200 mM
- Triton X-100	1% (w/v)
- Dithiothreitol (DTT)	10 mM
- Magnesium Chloride	70 mM
- β-Nicotinamide Adenine Dinucleotide (NAD)	2.5 mM
- poly (dIC)	300 ng / μL
Genomic DNA (DNase I digested)	100 ng / μL
OLA Probe Set:	
- First probe - CYC	5 nM
- First probe - RNA	5 nM
- Second probe	10 nM
Nuclease Free Water	

[0266] *Taq* Ligase was diluted to 2.0 units / μL in the 1 X OLA Buffer 2 Mixture. The volume of *Taq* Ligase was sufficient to form the following stock of

OLA reagent. The common working stock of OLA reagent was formed as specified in the following Table 3. The following volumes of components are based on a single 10 μL OLA reaction volume. Depending on the number of OLA reactions that are desired, one can form the particular volume of stock OLA reagent.

TABLE 3

OLA Reaction Component	**1X OLA Reaction Volume (μL)**	**X number of OLA Reactions = Total Volume (μL)**
10X OLA Buffer 2 Mixture	1.0	
Nuclease Free Water	5.4	
Taq DNA Ligase (2.0 units / μL)	0.6	

[0267] For each reaction with one of the two probe sets of Table 1, 7 μL of the stock OLA reaction composition of Table 3 was combined with 2.0 μL of the given probe set using the OLA probe set concentrations in Table 2, and 1.0 μL genomic DNA using the genomic DNA concentration in Table 2. The final assay component concentrations for the OLA reactions are set forth in Table 4 below.

TABLE 4

OLA Compon nt	Concentration
Thermus aquaticus (*Taq*) DNA Ligase	0.12 units / μL
Sodium (3-[N-Morpholino]propanesulfonate) (MOPS)	20 mM
Triton X-100	0.1 % (w/v)
Dithiothreitol (DTT)	1 mM
Magnesium Chloride	7 mM
β-Nicotinamide Adenine Dinucleotide (NAD)	0.25 mM
poly (dIC)	30 ng / μL
Genomic DNA (DNase I digested)	10 ng / μL
OLA Probe Set:	
- First probe - CYC	1 nM
- First probe - RNA	1 nM
- Second probe	2 nM

[0268] For these examples, each of the two different probe sets in Table 1 were included in different reactions for three different genomic DNA samples. Thus, there were six different reactions volumes, each with a different combination of probe set and genomic DNA sample. The three genomic DNA samples were obtained from Coriell Cell Repositories (Camden, NJ) and were designated as follows: NA17103, NA17212, and NA17247. Prior to combining each of the genomic DNA samples in the ligation reaction composition, the genomic DNA was fragmented by DNase I digestion.

[0269] The ligation reaction volumes were subjected to the reaction conditions shown in Table 5 below using an ABI 9700 Thermal Cycler (Applied Biosystems, Foster City, CA). The reaction volumes were kept on ice until they

were transferred to the thermal cycler. The OLA reaction tubes were transferred from ice to the thermal cycler when the thermal cycler reached the first hold temperature of 90°C.

TABLE 5

Step	Step Type	Temperature (°C)	Time
1	Hold	90	3 minutes
2	14 cycles	90 54	5 seconds 4 minutes
3	Hold	99	10 minutes
4	Hold	4	∞

C. Exemplary Amplification Reactions

[0270] A 10X primer / labeled probe composition was formed by combining the forward and reverse primers of Table 1 and the two TaqMan® probes labeled with VIC and FAM so that they were in final concentrations as follows:

Forward Primer 9 μM

Reverse Primer 9 μM

TaqMan® [VIC] 2 μM

TaqMan® [FAM] 2 μM.

[0271] Each PCR reaction volume included the following components:

12.5 μL -- 2X TaqMan® Universal PCR Mix (Applied Biosystems, Foster City, CA). The PCR Mix includes PCR buffer, dNTPs, $MgCl_2$, uracil-N-glucosidase, and Ampli*Taq* Gold® DNA polymerase (Applied Biosystems, Foster City, CA);

2.5 μL -- 10X primer / labeled probe composition discussed above;

8 μL -- water; and

2 μL -- OLA reaction volume after the ligation reaction from Example 1B above.

[0272] Thus, the total PCR reaction volume for each PCR reaction was 25 μL. Each PCR reaction volume was subjected to the reaction conditions shown in Table 6 below using an ABI 7700 Thermal Cycler (Applied Biosystems, Foster City, CA).

TABLE 6

Step	Step Type	Temperature (°C)	Time
1	Hold	50	2 minutes
2	Hold	95	10 minutes
3	40 cycles	92 60	15 seconds 1 minute

[0273] In Assay 1, the signal from the TaqMan® probe labeled with FAM indicated that the genomic DNA NA17103 was homozygous for the allele corresponding to the First Probe-RNA (1), which has a "C" as the nucleotide at the pivotal complement. Thus, the genomic DNA NA17103 correctly was determined to be homozygous at the locus analyzed in Assay 1 with "G" at the pivotal nucleotide.

[0274] In Assay 1, the signal from the TaqMan® probe labeled with VIC indicated that the genomic DNA NA17212 was homozygous for the allele corresponding to the First Probe-CYC (1), which has a "T" as the nucleotide at the pivotal complement. Thus, the genomic DNA NA17212 correctly was determined to be homozygous at the locus analyzed in Assay 1 with "A" at the pivotal nucleotide.

[0275] In Assay 1, the signals from the TaqMan® probes labeled with FAM and VIC indicated that the genomic DNA NA17247 was heterozygous for the alleles

corresponding to both the First Probe-CYC (1) and the First Probe-RNA (1). Thus, the genomic DNA NA17247 correctly was determined to be heterozygous at the locus analyzed in Assay 1 with "G" and "A" at the pivotal nucleotides.

[0276] In Assay 2, the signal from the TaqMan® probe labeled with FAM indicated that the genomic DNA NA17103 was homozygous for the allele corresponding to the First Probe-RNA (2), which has an "A" as the nucleotide at the pivotal complement. Thus, the genomic DNA NA17103 correctly was determined to be homozygous at the locus analyzed in Assay 2 with "T" at the pivotal nucleotide.

[0277] In Assay 2, the signals from the TaqMan® probes labeled with FAM and VIC indicated that the genomic DNA NA17212 was heterozygous for the alleles corresponding to both the First Probe-CYC (2) and the First Probe-RNA (2). Thus, the genomic DNA NA17212 correctly was determined to be heterozygous at the locus analyzed in Assay 2 with "T" and "C" at the pivotal nucleotides.

[0278] In Assay 2, the signal from the TaqMan® probe labeled with VIC indicated that the genomic DNA NA17247 was homozygous for the allele corresponding to the First Probe-CYC (2), which has a "G" as the nucleotide at the pivotal complement. Thus, the genomic DNA NA17247 correctly was determined to be homozygous at the locus analyzed in Assay 2 with "C" at the pivotal nucleotide.

[0279] Other assays that employed the same concentrations of materials and same thermal cycling conditions as assays 1 and 2, but that employed different probe sets to detect the presence or absence of two alleles at different loci, were also performed. Some of those assays resulted in false negative signal. It was concluded that the second probes of those probe sets were defective, which inhibited appropriate ligation.

[0280] Although the invention has been described with reference to certain applications, methods, and compositions, it will be appreciated that various changes and modifications may be made without departing from the invention.